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**Biochemical Methodology**

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**for the Assessment of**

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**Vitamin A Status**

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A Report of the  
International Vitamin A  
Consultative Group (IVACG)

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The purpose of the International Vitamin A Consultative Group (IVACG) is to guide international activities aimed at reducing vitamin A deficiency worldwide. The group offers consultation and guidance to various operating and donor agencies who are seeking to reduce vitamin A deficiency and its accompanying blindness. As part of this service, IVACG has prepared guidelines and recommendations for:

- Assessing the regional distribution and magnitude of vitamin A deficiency;
- Developing intervention strategies and methodologies to combat vitamin A deficiency;
- Evaluating effectiveness of implemented programs on a continuing basis so that the evaluation of the effectiveness of intervention techniques is a continuing and dynamic procedure;
- Research needed to support the assessment, intervention and evaluation of programs.

Monographs published by the International Vitamin A Consultative Group are:

- Guidelines for the Eradication of Vitamin A Deficiency and Xerophthalmia (1977);
- Recent Advances in the Metabolism and Function of Vitamin A and Their Relationship to Applied Nutrition (1979);
- The Safe Use of Vitamin A (1980);
- The Symptoms and Signs of Vitamin A Deficiency and Their Relationship to Applied Nutrition (1981).

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## CHAPTER I

# Introduction

In the past decade, the realization has grown that vitamin A deficiency—and the blindness that results from its acute form in young children—remains a major public health problem in many parts of the world.<sup>1,5</sup> In recent years extensive surveys of vitamin A malnutrition have been conducted in several countries, and various intervention strategies have been employed to improve the vitamin A nutritional status and to minimize the incidence of nutritional blindness.

Most of these surveys have employed a combination of clinical and biochemical criteria, both to evaluate the presence of a public health problem and to assess the impact of a given intervention. Considerable attention has recently been given to the clinical criteria of greatest utility.<sup>1,6</sup> The objective of the present manual is to describe commonly used biochemical methods for measuring vitamin A and provitamin A carotenoids in nutritional surveys, particularly in the light of recent important advances in instrumentation. Methods have been divided into two groups—those which are preferred or may be used internationally (Chapter IV)—and others which may be useful in special circumstances (Chapter V). We have not included procedures for the analysis of carotenoids in foodstuffs, as that is beyond the scope of this monograph. (See Chapter IX and reference 8 for a treatment of analytical methods for carotenoids.)

In the opinion of the authors, the most useful methods under given conditions are described in detail, together with comments on their use, necessary precautions to be observed, and their inherent limitations.

The necessary procedures and precautions in the collection and handling of biological specimens to assess accurately vitamin A-active substances by any of the biochemical methods are described (Chapter II).

The importance of using appropriate reference standards (Chapter III) and of instituting laboratory quality control (Chapter VI) is stressed, and the manner in which the data obtained might best be expressed and interpreted (Chapter VII) is carefully considered. Finally, the interrelationships among various indicators of vitamin A status are briefly discussed (Chapter VIII) and some recommendations for further studies given (Chapter X).

Although the methodology for vitamin A and carotenoids has been briefly considered in earlier publications of the International Vitamin A Consultative Group,<sup>2,3</sup> no specific laboratory manual of these methods for use in nutritional surveys has appeared since the classical ICNND manual of 1963.<sup>7</sup> That valuable work, now of course much dated, employed a useful format which we have modified only slightly in the present volume.

Many improvements in methodology have occurred during the past 18 years. The characterization of retinol-binding protein in the plasma has stimulated the development of sensitive assays for its measurement. Extensive quantitative use has been made of the fluorescent properties of retinol. The advent of high pressure liquid

chromatography (HPLC) and the availability of packed columns of tiny, uniformly coated particles of silica have enormously increased the resolution of adsorption chromatography while greatly reducing the time of analysis. Biological samples other than blood, and most particularly milk and liver, have been used for survey purposes. And some of the older methods, such as the Bessey-Lowry UV inactivation procedure for serum and the colorimetric (Carr-Price) methods, have been modified and improved.

The major dilemma which faced the authors in developing this manual was to find a suitable compromise between the best techniques available for survey use and those which require the simplest instrumentation. For example, high pressure liquid chromatography is clearly the method of choice for both vitamin A and carotenoid analyses. Since these instruments can be effectively used for a wide range of biochemical assays of nutritional significance, all leading nutrition research institutions should have them. But at present they are expensive to purchase and maintain, and clearly are not available to nutritional scientists in many parts of the world. At the opposite extreme, the colorimetric methods can be done cheaply with a simple colorimeter, which is available almost everywhere. But unfortunately, colorimetric methods suffer from a variety of inherent difficulties that significantly reduce their reliability. Although some of us argued that the colorimetric methods should not be included at all in the manual, the majority felt that a careful description of the pitfalls and the necessary precautions to be taken in using the method would be of service to that significant group of nutritional scientists whose methodological options are severely constrained.

A brief explanation of the format used in this manual is in order. In most sections, only those references relevant to the topic discussed are provided at the beginning of the section. In the text, only the references for that section are cited. Although redundancy is introduced by this procedure (i.e., some papers are referenced in many sections), having the citations for a given method right at hand is clearly much more convenient than having to search for a given reference in a general bibliography. Tables and figures, on the other hand, are numbered sequentially throughout the manual.

The dilemma of selecting units of concentration which are both scientifically acceptable and generally used is ever with us. Although we have come far from the yellow and blue Lovibund units of the late 1920s, the expression of concentrations of vitamin A and carotenoids in standard international (SI) units (e.g., moles per liter) is not common in the nutritional literature. Furthermore, when mixtures of compounds are present or a biological activity is expressed, irrespective of the compounds present, the use of SI units is either inappropriate or awkward. As a consequence, the authors have opted to use common parlance for concentrations of vitamin A and carotenoids (e.g.,  $\mu\text{g}/\text{dl}$  for plasma and milk, and  $\mu\text{g}/\text{g}$  for liver and other tissues). Unless otherwise indicated, these values are expressed in terms of retinol and beta-carotene, regardless of the specific compounds present. Retinol-binding protein (RBP) in plasma, on the other hand, is given in  $\text{mg}/\text{dl}$ .

In a similar vein, the absorbancies of pure vitamin A and beta-carotene are expressed as  $E_{1\%}^{1\text{cm}}$  values, which are so commonly employed in the nutritional literature, rather than as the formally more correct molecular extinction coefficients. While sympathizing with the purist who might wince at such usage, we concomitantly feel that the nutritionally-oriented user of this manual should above all feel comfortable with the units employed.

Although the methods have been described in adequate detail in this manual for use by skilled technicians familiar with the instrumentation employed in a given assay, the users are urged as well to refer to the original description of the procedure cited in the

references.\* If the present manual serves in a small way to improve the accuracy and reliability of biochemical indices of vitamin A status reported in the literature, its contributors will feel amply rewarded.

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1. World Health Organization. (1976) *Vitamin A Deficiency and Xerophthalmia*. WHO Technical Report Series No. 590, Geneva.
2. International Vitamin A Consultative Group. (1976) *Guidelines for the Eradication of Vitamin A Deficiency and Xerophthalmia*. New York: The Nutrition Foundation.
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5. World Health Organization. (1982) *The Control of Vitamin A Deficiency and Xerophthalmia*. WHO Technical Report Series No. 672, Geneva.
6. International Vitamin A Consultative Group. (1981) *The Symptoms and Signs of Vitamin A Deficiency and Their Relationship to Applied Nutrition*. New York: The Nutrition Foundation.
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8. DeRitter, E. and Purcell, A. E. (1981) Carotenoid analytical methods. In: *Carotenoids as Colorants and Vitamin A Precursors*. J. C. Bauernfeind, ed., New York: Academic Press.

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\*A set of selected references is available through The Nutrition Foundation in Washington, D.C., *Reprints of Selected Methods for the Analysis of Vitamin A and Carotenoids in Nutrition Surveys*, July 1982.

## CHAPTER II

# Collection and Handling of Biological Specimens

## General Considerations

### References

1. Bessey, O. A., Lowry, O. H., Brock, M. F., and Lopez, J. A. (1946) The determination of vitamin A and carotene in small quantities of blood serum. *J. Biol. Chem.* 166:177.
2. Parkinson, C. E. and Gal, I. (1972) Factors affecting the laboratory management of human serum and liver vitamin A analyses. *Clin. Chim. Acta* 40:83.
3. Kahan, J. (1966) A method for the fluorometric determination of vitamin A. *Scand. J. Clin. & Lab. Invest.* 18:679.
4. Utley, M. H., Brodovsky, E. R., and Pearson, W. N. (1958) Hemolysis and reagent purity as factors causing erratic results in the estimation of vitamin A and carotene in serum by the Bessey-Lowry method. *J. Nutr.* 66:205.

### Principle

Vitamin A and its precursor carotenoids in biological specimens are labile substances requiring special handling to avoid losses prior to analysis. Some precautions are specific to the method of analysis employed and will be discussed in subsequent chapters in relation to that specific procedure. Others are more general and must be observed to minimize potential extrinsic sources of error. The four most critical general factors are:

- *Oxidation.* Vitamin A and carotenoids are readily destroyed by oxidation in the presence of air. Heat and the presence of metal ions (e.g., iron and copper) potentiate this phenomenon.<sup>1,2</sup>
- *Exposure to light.* Vitamin A and carotenoids are destroyed by exposure to light. These compounds undergo structural phototransformations particularly when exposed to light in the ultraviolet region (below 350 nm). Although sunlight is more destructive than artificial light, direct exposure to both should be avoided.<sup>2</sup>
- *Hemolysis.* Erroneous results are obtained by several analytic procedures when applied to hemolyzed samples.<sup>1,4</sup> Thus, hemolysis must be avoided.
- *Extraction.* Complete extraction of vitamin A into an organic solvent will occur only after complete precipitation of protein by the addition of alcohol in the appropriate ratio (approximately 1:1 v/v). On the other hand, an aqueous solution too concentrated in ethanol will inhibit the extraction of vitamin A and carotenoids into the organic solvent phase.

# Blood

## References

1. International Vitamin A Consultative Group. (1979) *Guidelines for the Eradication of Vitamin A Deficiency and Xerophthalmia: Recent Advances in the Metabolism and Function of Vitamin A and Their Relationship to Applied Nutrition*. New York: The Nutrition Foundation.
2. Parkinson, C. E. and Gal, I. (1972) Factors affecting the laboratory management of human serum and liver vitamin A analyses. *Clin. Chim. Acta*, 40:83.

## Principle

Over 95% of the vitamin A normally found in fasting blood is bound to its carrier protein, retinol-binding protein (RBP). Only after ingestion of a meal rich in vitamin A, after the administration of a high dose of vitamin A, or in certain disease states (vitamin A toxicity, severe liver disease, severe protein-calorie malnutrition) does one find elevated circulating levels of lipoprotein-bound retinyl esters. In well-nourished populations, blood levels of vitamin A range from 20-70  $\mu\text{g}/\text{dl}$  with most individuals having values above 36  $\mu\text{g}/\text{dl}$ .

Once blood is removed from the body, vitamin A, as well as carotenoids, are susceptible to losses that can be minimized by rapid separation of serum or plasma from red blood cells; protection from light; handling in an oxygen-free atmosphere, such as under nitrogen; and immediate analysis or freezing. Since survey circumstances do not always allow ideal conditions to be met, alternative procedures have been devised that minimize extrinsic error in estimating true vitamin A levels. Preferred and alternative procedures, as well as improper techniques, are summarized in Table 1.

TABLE 1.  
Collection and Handling of Blood Samples

Preferred Procedure	Acceptable Alternatives	Improper Procedures
<i>Blood collection</i> Venous blood in containers permanently labeled	Capillary blood permanently labeled	Milking or squeezing the finger or other tissue from which blood is being drawn. Use of water-soluble ink and labels.
Blood without anticoagulant for obtaining serum <sup>a</sup>	Plasma can be used	Use of hemolyzed blood
Centrifuge immediately after clotting	Cool whole blood to 1-4 C, replace air with an inert gas, stopper, and ship to the laboratory. Avoid hemolysis. Do not freeze. Upon arrival, centrifuge immediately. Prevent warming of the specimen.	Centrifuging more than 24 hours after blood collection
Analyze serum immediately after centrifugation	Store at -20 C or lower. When possible, measure the exact aliquots to be used and freeze them. Minimize air head space. When feasible, displace the air atmosphere in the tube by an inert gas.	Repeated freezing and thawing

<sup>a</sup>Vacutainer with disposable needles is recommended.

## Procedure

### *Preferred Procedure*

Glass vacutainer tubes without anticoagulant and fitted with disposable needles are used to draw the required amount of blood (depending on the method of analysis) from the antecubital vein. All precautions should be taken to clean properly the skin surface before drawing blood. A tourniquet of soft rubber tubing is applied to the upper arm shortly before inserting the needle and is released soon after the blood begins to flow into the tube. This procedure will maximize the opportunity for successfully obtaining a blood specimen and will minimize the risk of hemolysis and of producing a hematoma. Plastic tubes and rubber stoppers of poor quality often contain contaminants that interfere with certain analytical procedures. Consequently, any such items which are used should be of high quality and before being routinely employed should be tested both under the specified assay conditions and under the exact conditions of extraction to be used. This precaution is of particular importance when a fluorescent assay is used. It is preferable to collect blood under fasting conditions, although ingestion of breakfast might not substantially alter serum levels of retinol, total carotenoids, or RBP.

Special precautions should be taken to label specimens appropriately and permanently. The label should contain all the essential identifying information clearly written with indelible ink on labels secured to tubes by water-insoluble adhesive. Transportation in ice chests may bring specimen tubes in contact with moisture causing blurring of label information or loss of the label if these precautions are not taken. Placing the test tubes inside a sealed, water-tight container is therefore recommended.

Immediately after clotting (15-30 minutes at room temperature) the serum should be separated by centrifugation at 600-1000  $\times$  g for 10 minutes. A refrigerated centrifuge is recommended although not strictly necessary. Under survey conditions, if electricity is not available, a portable generator can be used for running the centrifuge. A hand-driven centrifuge is another alternative. Care must be exercised not to leave any blood cells in the serum that could subsequently hemolyze. If blood cells remain in the separated serum, repeat the centrifugation procedure. Transfer the sera to glass tubes of approximately the same volume as the serum available (to minimize the head space\* of air), then seal. Where possible, the head space should be filled with an inert gas such as nitrogen, argon or carbon dioxide of high purity. However, specimens kept frozen at  $-20^{\circ}\text{C}$  for up to six months with a minimal head space have proven to be stable.

When it is not possible to analyze the sera immediately, for example in a field station, the specimens should be properly labeled, flushed with nitrogen for 30-60 seconds, tightly capped, and placed in an ice chest for transportation to the base laboratory. A small, portable nitrogen cylinder can be readily transported to most survey sites. If possible, analyses should be performed soon after arrival at the laboratory. Specimens should be protected from exposure to air, and from light and warming from the time of collection to the completion of the analyses.

### *Alternative Procedures Acceptable Under the Following Circumstances*

*Difficulty in obtaining venous blood.* Capillary blood can be used when venous blood cannot be obtained for cultural or other reasons. Capillary blood is collected in capillary tubes after pricking the properly cleaned finger, ear lobe or other suitable part of the

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\*Head space is defined as the air space above the meniscus of the fluid collected and the cap or stopper. Generally, this space should be kept to a minimum volume by using suitably sized tubes. Replacement of air with an inert gas, as recommended above, is also highly advisable.

body. The blood should flow freely from the tissue. Do not milk or squeeze the finger or tissue since this may increase the risk of hemolysis and may dilute the specimen with interstitial fluid.

The blood should be centrifuged and separation of the serum performed immediately. If this is not possible, the capillary tubes may be transported to the laboratory maintained at all times in an upright position inside a container in an ice chest. When several capillaries are obtained from one person, these should be put together inside a larger, properly labeled tube. This minimizes risk of breakage and confusion in sample identification.

*Collection of plasma rather than serum.* Serum is preferred for the analysis because it maintains its homogeneity over time. Plasma, on the other hand, particularly after being stored frozen, may form a slight precipitate which can interfere with subsequent pipetting and with the quantitative analysis of RBP. However, total vitamin A values are the same whether serum or plasma is used.

*Delay under field conditions in the immediate separation of serum.* In the case of unavoidable delay in centrifuging blood, place the blood samples in an insulated chest with wet ice or coolant, replace the air with nitrogen, seal with high quality stoppers, transport to the laboratory, and separate the serum soon after arrival. The time between blood collection and serum separation should not exceed 24 hours. Special care must be taken to prevent hemolysis during transportation, particularly when traveling on rough roads where shaking becomes critical. This risk may be minimized by suspending the chest of specimens in the vehicle. Partial or total freezing of the whole blood specimens during storage or transportation, which will hemolyze the red blood cells, must be carefully avoided.

*Delay in transport of the serum to the laboratory.* Serum specimens can be stored under an inert gas at  $-8^{\circ}\text{C}$  (freezer compartment of a regular refrigerator) for up to five days. Then, the specimens, while maintained in a frozen state, should be transported to the laboratory for immediate analysis. Samples should be thawed at room temperature ( $20\text{-}25^{\circ}\text{C}$ ), and the specimen mixed well before taking aliquots. In surveys in remote areas where electricity or freezers are unavailable, liquid nitrogen can be used to maintain blood sera frozen. Liquid nitrogen containers, such as those used by veterinary laboratories in artificial insemination programs, are suitable for preservation and transport of human sera for up to two weeks. These containers have a capacity of seven gallons of liquid nitrogen and are equipped with six internal canisters, each of which can hold 21 test tubes ( $10 \times 75$  mm).

*Delay in analysis.* Serum specimens can be stored at  $-20^{\circ}\text{C}$  for up to six months without substantial loss of vitamin A. The length of safe storage, however, will depend on the exclusion of oxygen and on the analytical method to be used, as noted under specific procedures given in Chapters IV and V. It has been reported that some colorimetric techniques may give spurious values when applied to stored samples even after short periods of storage. Others have not confirmed this finding with their handling and storage procedures. Laboratories using colorimetric procedures should confirm the safe storage time under their specific conditions.

The specimens should be stored in tubes under an inert gas or with a minimum of head space. To minimize pipetting transfers, duplicate aliquots of the same volume to be used in analysis can be stored for short time intervals. The air atmosphere should be displaced by blowing in an inert gas such as nitrogen, carbon dioxide, or argon. If these latter gases are not of high purity (i.e., if they contain some oxygen), just minimizing the head space

and the gas/liquid interfacial area will reduce oxidative loss. Repeated thawing and refreezing should be avoided, since this will affect both the vitamin A concentration and the concentration of retinol-binding protein. When a delay in analysis is anticipated, a control serum of known concentration should be stored under the same conditions and analyzed concurrently with the unknown specimens. The values of the control serum should be within two standard deviations of the preestablished mean. This procedure will permit the quality of the stored samples to be checked (Chapter VI).

## Liver

### References

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3. Suthutvoravoot, S. and Olson, J. A. (1974) Plasma and liver concentrations of vitamin A in a normal population of urban Thai. *Am. J. Clin. Nutr.* 27:883.
4. Parkinson, C. E. and Gal, I. (1972) Factors affecting the laboratory management of human serum and liver vitamin A analyses. *Clin. Chim. Acta* 40:83.

### Principle

Liver is the major storage organ for vitamin A. In a normal, well-nourished adult population, over 90% of the total body reserve of vitamin A is found in the liver, almost entirely in ester form. When liver reserves of vitamin A are very low ( $<10 \mu\text{g/g}$ ), however, the kidney and other organs contribute significantly to the total body reserves. Nonetheless, the analysis of liver provides information about vitamin A status not obtainable in other ways.

The distribution of vitamin A in human liver is heterogeneous,<sup>1,2</sup> with the right lobe usually containing higher concentrations than the other lobes, and the concentrations at specific sites within a given lobe varying considerably. If single samples of at least 5 g are taken from the central part of the right lobe, however, values obtained are useful as an indicator of total body reserves of vitamin A.

### Procedure

Liver samples may be collected at autopsy. Using surgical gloves and other hygienic precautions, a 10-20 g sample of liver is excised from the middle portion of the right lobe. The sample is briefly blotted on a paper towel, wrapped in foil or placed in a plastic or glass container, permanently labeled, sealed, and placed in a closed ice chest for transport to the laboratory. Preferably, a portion of the sample is immediately analyzed; otherwise the sample is placed in a deep-freeze ( $-20^\circ\text{C}$  or lower) and stored frozen, preferably under nitrogen, until the analysis can be conducted.

Fortunately, vitamin A in liver samples is very stable.<sup>3</sup> In fresh pieces of liver stored at room temperature, vitamin A values do not change appreciably until autolysis is pronounced ( $>48$  hours). Vitamin A in pieces of liver is even more stable when stored in the frozen state; no changes in vitamin A values were noted, for example, in pieces of liver samples stored at  $-20^\circ\text{C}$  for 24 weeks.<sup>3</sup> After homogenization in water or buffer, however, vitamin A in fresh liver samples declined 7.6%, and in previously frozen samples declined

64% when stored at 4°C for one week. Thus, although immediate analysis is always preferable, pieces of fresh liver samples may be stored for a few days in the dark under refrigeration before analysis, whereas pieces of frozen samples should be analyzed immediately upon thawing. Do not homogenize specimens until ready to analyze.

Even in sealed containers, some dehydration of the surface of frozen liver samples occurs, and some blood oozes out and freezes. Thus, frozen samples should be trimmed and blotted before analysis.

## Breast Milk

### References

1. Arroyave, G., Aguilar, J. R., Flores, M., and Guzman, M. A. (1979) *Evaluation of Sugar Fortification with Vitamin A at the National Level*. PAHO Scientific Publication No. 384, Washington, D.C.
2. World Health Organization. (1965) *Nutrition in Pregnancy and Lactation*. WHO Technical Report Series No. 302, Geneva.

### Principle

Normally, breast milk of well-nourished women contains an average of about 50 µg/dl.<sup>2</sup> Ideally, the total quantity of milk from a full breast should be collected, gently but thoroughly mixed, the volume measured, and suitable aliquots taken for analyses. In field surveys, however, this is impracticable. An acceptable alternative is the collection of a small sample under specified conditions. Since the concentration of some milk components, especially lipids and liposoluble substances, such as vitamin A, increases during the suckling period (Chapter VII), the procedure and conditions for the collection of the milk sample must be standardized. One procedure is to ask the lactating mother to collect 8-10 ml of milk by manual self-expression from her full breast *before* the baby starts suckling. In a large study carried out in rural Guatemala this procedure was found to be feasible and convenient and was well received by the women.<sup>1</sup> If the conditions for obtaining the sample as described are not consistently met, the interpretation of milk retinol values becomes difficult. In any case, the actual collection procedure and conditions used must be reported. In addition, whenever possible, the total breast contents should be obtained from a subsample of lactating women under study. The average vitamin A concentration in the total breast contents can then be compared with that obtained in the 10 ml sample obtained as outlined above.

### Procedure

Glass test tubes (10 ml) with rubber stoppers\* are used. After collection, the samples are immediately placed in an insulated box with ice for transportation from the field to the laboratory. Ideally, aliquots for analysis should be prepared immediately from a well-mixed specimen. If not analyzed immediately, the aliquots may be stored frozen at -20°C or lower. The stability of vitamin A under these conditions is at least as good as that for blood serum. If the full sample is frozen, the thawed sample must be thoroughly mixed before pipetting aliquots. A vortex mixer is usually employed for this purpose. Since prolonged freezing often causes profound separation of lipid and aqueous phases, homogenization in an all glass apparatus or sonication may be required. The same

\*High quality rubber stoppers (as used for vacutainer tubes) should be employed.

precautions described for serum specimens should be observed, i.e., avoid exposure to light, warming, repeated freezing and thawing, and opportunities for oxidation.

## CHAPTER III

# Reference Standards

### References

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Both vitamin A and beta-carotene are inherently unstable substances. Both easily decompose in the presence of oxygen and light, particularly ultraviolet light. In many instances, polyenes are assayed in laboratories equipped with incandescent rather than fluorescent lighting to reduce the amount of ultraviolet absorption. The ultraviolet component of fluorescent light can also be restricted by passage through transparent yellow paper or plastic. Contact between the light and the filtering material must, of course, be avoided to minimize the hazard of fire. Ideally, assays should be done under red or filtered light. In the spectrophotometric assays cited below, the spectrophotometer used should be calibrated before analyzing standards of vitamin A or beta-carotene.

### Vitamin A

Vitamin A is provided as a USP Reference Standard. The  $E_{1cm}^{1\%}$  value for all *trans*-retinol in hexane or ethanol at 325 nm is taken as 1850<sup>1</sup> throughout this manual (Table 2). Since the absorbancy of the esters in hexane is the same, on a molar basis, as that of retinol,<sup>2</sup>  $E_{1cm}^{1\%}$  values for the esters in hexane are calculated on the basis of their relative molecular weights. In ethanol or isopropanol, however, the molar absorbancy of the esters is 3-4% lower than that of free retinol.<sup>2</sup>

The all-*trans* vitamin A standard is currently available from the U.S. Pharmacopeial Convention, Inc. (USPC), 12601 Twinbrook Parkway, Rockville, Maryland, 20852. The standard is provided in lots of 24 capsules, each capsule containing approximately 250 mg of a solution of vitamin A acetate in cotton seed oil. Each gram of the solution contains close to 34.4 mg of all-*trans* retinyl acetate, which is equivalent to 30.0 mg of all-*trans* retinol or 100,000 IU vitamin A. The actual potency of capsules varies somewhat, depending upon the batch from which they are derived. The assayed vitamin A content is provided by the producer on the label for each batch. Just before use, the value given should be checked spectrophotometrically using the extinction coefficient noted.

The capsules should be stored in a cool, dry place protected from light. Once a capsule is opened, the unused material should be discarded. Unopened capsules held beyond the

expiration date noted on the container should be checked spectrophotometrically before being used as a standard.

Other biochemical supply companies furnish vitamin A acetate in cotton seed oil. These products are secondary standards derived primarily from the USPC standards. The actual concentration of vitamin A acetate in these preparations may vary plus or minus 5%. In addition, commercially available crystalline forms of all-*trans* retinol and retinyl acetate may be used for preparing standards in one's own laboratory. For their assay, however, a high quality UV/VIS spectrophotometer must be available.

TABLE 2.  
E<sub>1cm</sub><sup>1%</sup> Values for Vitamin A and Beta-Carotene

Compound	$\lambda$	Hexane	Ethanol
All- <i>trans</i> retinol	325	1850	1850
All- <i>trans</i> retinyl acetate	325-328	1610	1565
All- <i>trans</i> beta-carotene	450-452	2550	2375

## Carotene Reference Standards

The E<sub>1cm</sub> value for crystalline all-*trans* beta-carotene in hexane at 452 nm is 2550<sup>34</sup> and in ethanol is somewhat lower (Table 2). Ampules containing pure all-*trans* beta-carotene can be obtained from a number of companies, such as Sigma Chemical Company and Nutritional Biochemical Company. The ampules thus furnished are approximately 95% beta-carotene. The actual concentration of beta-carotene may vary rather widely, however. Since carotene is very unstable, it is not unusual to find a considerable amount of deterioration in these materials. When this occurs, it is often difficult to solubilize the sample in organic solvent fully. Isomeric changes may also have taken place. For these reasons, beta-carotene solutions must be checked spectrophotometrically before use as reference standards. If significant deviation from the expected value is observed, analysis by HPLC or TLC is recommended. When appropriate, instructions for preparing stock and standard solutions of beta-carotene are included under specific methods.

## Selected Wavelengths for Analysis

In methods reported over the past thirty years, including those summarized in this manual, the original publications often cite wavelengths for the analysis of vitamin A or carotenoids which differ from those given above, e.g., 325-330 nm for retinol and its esters and 452 ± 5 nm for beta-carotene or mixed carotenoids. These discrepancies might be due to a slight shift in the  $\lambda$  max due to the solvent used, to other trace absorbing substances in an extract or solvent, to error in the calibration of the instrument, or to the presence of some isomers of retinol or beta-carotene in the standard or biological extract. For most purposes, these discrepancies are of trivial importance, inasmuch as the absorption spectra of these substances is broad enough near the  $\lambda$  max to give essentially the same absorbancy over a 5 nm range. Consequently, the originally selected wavelength in a given method is often cited rather than those given above.

## Preparation of Laboratory Standard Solutions of Vitamin A Using the USP Reference Standard

### *Procedure 1*

Weigh approximately 30 mg of the oily solution of vitamin A (which contains approximately 34.4 mg retinol/g) and dissolve in 100 ml ethanol (the solubility of the oil in ethanol does not permit more concentrated solutions). This solution can be used as a stock if kept in a dark bottle and refrigerated. If a calibration curve is desired, prepare dilutions of the stock standard in ethanol and carefully determine the absorbance of these solutions. Depending on the particular method selected, stock and working standard solutions can be prepared in specified solvents such as hexane or a 1:1 xylene-kerosene mixture.

### *Procedure 2*

Weigh approximately 30 mg of the oily solution of vitamin A and dilute with chloroform up to 100 ml. Since vitamin A is less stable in chloroform than in ethanol, this solution must be used immediately and its concentration must be determined just before each set of assays. Pipette 50  $\mu$ l of this solution into a clean tube and evaporate the chloroform. The tube, capped under a nitrogen-filled head space, can be stored for weeks at  $-20^{\circ}\text{C}$  in the dark. Just prior to use, carefully add 3 ml ethanol to 2-4 tubes and determine the concentration of retinol in the manner described above. Working standards can be prepared by dissolving the contents of other tubes of the same set in an appropriate amount of ethanol (3 ml), or in other specified solvents as required.

This second method is especially suited for sending retinol standards to laboratories which lack spectrophotometric facilities. The stability of the vitamin A standard under the precisely employed conditions of preparation, transport and storage should be checked before the procedure is routinely used.

## CHAPTER IV

# Preferred Methods for Vitamin A

## High Pressure Liquid Chromatography (HPLC) and Ultraviolet Absorbance

### References

1. DeRuyter, M. G. M. and DeLeenheer, A. P. (1976) Determination of serum retinol (vitamin A) by high-speed liquid chromatography. *Clin. Chem.* 22:1593.
2. Bieri, J. G., Tolliver, T. J., and Catignani, G. L. (1979) Simultaneous determination of  $\alpha$ -tocopherol and retinol in plasma or red cells by high pressure liquid chromatography. *Am. J. Clin. Nutr.* 32:2143.
3. Bridges, C. D. B., Fong, S. L., and Alvarez, R. A. (1980) Separation by programmed-gradient high-pressure liquid chromatography of vitamin A isomers, their esters, aldehydes, oximes and vitamin A<sub>2</sub>: Presence of retinyl ester in dark-adapted goldfish pigment epithelium. *Vision Res.* 20:355.
4. Paanaker, J. E. and Groenendijk, G. W. T. (1979) The separation of geometric isomers of retinyl ester, retinal and retinol, pertaining to the visual cycle, by high-pressure liquid chromatography. *J. Chromatogr.* 168:125.

### Principle

A given volume ( $\sim 100 \mu\text{l}$ ) of serum or plasma is diluted with ethanol or methanol, which denatures plasma proteins, and the vitamin A is extracted with a suitable organic solvent. After centrifugation, an aliquot of the organic phase is injected onto a normal or reversed phase HPLC column, followed by an eluting solvent of suitable polarity. Retinol, which is eluted as a sharp peak within 1-6 minutes, is detected by a sensitive UV detector set at 325-328 nm. Retinol is quantitated by use of peak height ratios or peak area ratios relative to an internal standard (retinyl acetate or other appropriate analogs).

### Apparatus

Analytical microbalance

Ultraviolet-visible spectrophotometer

Centrifuge, desk top

Vortex mixer

Rotary evaporator

High performance liquid chromatography system, equipped with a variable wavelength detector or suitable filters

Nitrogen or other inert gas

Water bath, 40-50°C, constant temperature

Micropipettes 10  $\mu\text{l}$ , 15  $\mu\text{l}$ , 50  $\mu\text{l}$ , 100  $\mu\text{l}$

Glass test tubes, 10  $\times$  75 mm or conical centrifuge tubes with polyethylene screw caps

Pasteur pipettes  
Hamilton 710 syringe or a similar product

## Reagents

Retinyl acetate, all-*trans* (highest purity)  
Retinol, all-*trans* (highest purity)

All organic solvents should be checked for absorbancy at the detection wavelength and purified by distillation or by passage through a silicic acid column before use. Solvents used for HPLC must be degassed and free of particles.

### *Procedure 1*

Petroleum ether (bp 40-60°C), redistilled  
Absolute methanol, analytical grade  
Isopropanol, analytical grade  
Dichloromethane, analytical grade

### *Procedure 2*

Hexane, nanograde\*  
Anhydrous ethyl ether, analytical grade  
Absolute methanol, analytical grade  
Absolute ethanol, analytical grade

## Procedures

### *Procedure 1 (DeRuyter and DeLeenheer)*

Transfer 100  $\mu$ l of serum (or plasma), 15  $\mu$ l of internal standard solution [ $\sim 4 \mu$ g/ml of retinyl acetate or propionate or of all-*trans* 9-(4-methoxy-2,3,6 trimethylphenyl)-3,7-dimethyl-2,4,6,8-tetraenol] and 100  $\mu$ l of methanol to a conical centrifuge tube (7.6  $\times$  0.9 cm). Mix the contents of the tube with a vortex-type mixer. Add 200  $\mu$ l of extraction solvent (petroleum ether:dichloromethane:isopropanol, 80:19.3:0.7, respectively by volume) and cap the tube (polyethylene cap). Extract by interrupted mixing on the vortex-type mixer for 60 seconds. After centrifugation (3000 rpm, 2 minutes) inject 100  $\mu$ l of the supernate on top of the column by use of a Hamilton 710 syringe, 100  $\mu$ l capacity. Elute with the same solvent as used for extraction.

### *Procedure 2 (Bieri, Tolliver and Catignani)*

Pipette 100  $\mu$ l plasma (or serum) and 100  $\mu$ l of the internal standard solution in ethanol (0.4-0.6  $\mu$ g retinyl acetate/ml) into a 6  $\times$  50 mm glass test tube. Mix the contents vigorously on a vortex-type mixer for 10-20 seconds. Add 120  $\mu$ l spectrograde† hexane and mix the contents vigorously but intermittently for 45 seconds on a vortex mixer, making sure that the bottom layer is thoroughly extracted. After centrifugation (2200 rpm, 5 minutes) to ensure phase separation, carefully transfer 75  $\mu$ l of the upper hexane layer to a 3 or 5 ml conical centrifuge tube. If some of the lower phase is drawn into the pipette, abnormal results will be obtained. Evaporate the solvent, preferably under a stream of nitrogen, in a 60°C water bath. Be sure that water vapor does not condense on the inside of the tube during evaporation or cooling. Immediately dissolve the lipid

\*A trade term for very highly purified hexane

†Hexane containing very few contaminants which absorb light in the UV/VIS range.

residue in 25  $\mu$ l diethyl ether followed by 75  $\mu$ l methanol. Gently swirl and tap to enhance solubility of the lipid residues. Inject about 90  $\mu$ l of the solution on the column with a 100  $\mu$ l syringe. Elute with filtered (0.5 $\mu$  filter) methanol: water (95:5).

### *Chromatographic Conditions\**

	<b>Procedure 1</b>	<b>Procedure 2</b>
Column	15 $\times$ 0.2 cm i.d. MicroPak Si-10 petroleum ether/dichloromethane/isopropanol (80/19.3/0.7, by vol)	30 $\times$ 0.39 cm i.d. micro Bondapak C-18† methanol/water (95/5, by vol)
Flow rate	0.5 ml/min	2.5 ml/min
Pressure	10 kg/cm <sup>2</sup>	Not specified
Detector wavelength	328nm	328nm for retinol; 280 nm for retinol and alpha-tocopherol
Detection sensitivity	0.04 AUFS†† on recorder	0.01 AUFS†† on recorder
Temperature	Ambient	Ambient
Recorder	Not specified	10mv, 1 cm/min
Elution time:		
Retinol	5.0 min	2.2 min
Internal standard	6.2 min	3.0 min

\*Other equivalent columns and conditions may be used.

† A guard column ( $\approx$  22 mm) containing Bondapak C-18 Corasil is strongly recommended.

†† AUFS: Absorption units full scale

### *Analysis by the Peak Height (or Area) Ratio Procedure*

By use of an internal standard, losses due to incomplete extraction, inaccurate aliquots, oxidation, etc. are automatically corrected, provided that the internal standard has physical and chemical properties sufficiently similar to retinol, is suitably separated from retinol on HPLC, does not coincide with other 325nm absorbing materials in serum, and is not converted to retinol under the assay conditions. A precisely known amount of the internal standard is added to the aliquot of plasma to be analyzed. By determining the relative extraction efficiency and detector response of retinol and the internal standard, a standard curve is fashioned in which the ratio of peak heights (or areas) is plotted against the retinol concentration in plasma. In experimental samples, the peak height (or area) ratio is determined and the appropriate plasma retinol concentration determined from the standard curve or regression line formula.

A standard curve is prepared by adding varying amounts of retinol (i.e., 10, 20, 40, 80 and 120 ng) to a fixed amount (i.e., 50 ng) of internal standard in a final volume of 100  $\mu$ l of eluting solvent, injecting the solution on HPLC under assay conditions, measuring the

peak heights, and calculating the peak height ratio. The peak height ratio is then plotted as the abscissa with the plasma retinol concentration (for a 100  $\mu$ l plasma aliquot) as the ordinate.

A more accurate procedure is to add different ratios of retinol and of the internal standard to a 100  $\mu$ l sample of vitamin A-free plasma, obtained either from a vitamin A-deficient animal or by irradiating a serum sample for  $\sim$ 3 hours with longwave UV light ( $>$ 320 nm). The entire extraction procedure is then performed and the standard curve fashioned as indicated above.

If an integrator is available, the ratio of peak areas of retinol and the internal standard at different relative concentrations can be similarly plotted as the abscissa vs. the plasma retinol concentration (for a 100  $\mu$ l aliquot) as the ordinate.

### Standards

All-*trans* retinyl acetate in a dry crystalline form is reasonably stable in the dark under an inert gas at low temperatures.  $E_{1\%}^{1\text{cm}}$  values in ethanol at room temperature for all-*trans* retinyl acetate at 328 nm is 1565, and for all-*trans* retinol at 325 nm is 1850. When ethanol is used as the solvent, the  $E_{1\%}^{1\text{cm}}$  value for retinol in the form of retinyl acetate would be 3% less, or 1795. A stock standard solution of retinyl acetate in ethanol (50  $\mu$ g retinol/ml) is prepared by dissolving about 1 mg of retinyl acetate in 10 ml of ethanol, determining the concentration in a 1:30 dilution of an aliquot in ethanol by use of the above  $E_{1\%}^{1\text{cm}}$  at 325 nm, and then diluting the stock standard appropriately with ethanol. A sample calculation follows:

A 1:30 dilution of a solution of all-*trans* retinyl acetate, prepared and assayed as indicated above, gave an absorbancy reading at 328 nm of 0.58 in ethanol. The concentration of vitamin A, expressed as retinol, in this solution is:

$$\frac{0.58 \times 30}{0.1795\text{M}} \quad \text{or} \quad 96.9 \mu\text{g/ml.}$$

The 10 ml of this solution is then diluted to 19.4 ml to yield the stock standard with 50  $\mu$ g retinol/ml.

This stock standard solution is stable for more than a week when kept at 4°C in the dark, but should be checked periodically for deterioration both by HPLC and by spectra. A working standard, i.e., internal standard solution, is prepared at least weekly by appropriate dilution of the stock solution. If the crystalline retinyl acetate is impure, purification by HPLC should precede the preparation of standards.

Retinol is prepared by saponifying retinyl acetate with alcoholic KOH in the presence of 5% pyrogallol, extracting into hexane and purifying by chromatography on a column of weakened (6.5% water) alumina (p. 37).

The column is developed by flushing with 10 ml hexane followed by 10 ml 2% ethyl ether in hexane (v/v) to elute any residual retinyl ester. The retinol is eluted by 50% ethyl ether in hexane (v/v) and the concentration measured spectrophotometrically.

In place of all-*trans* retinyl acetate as an internal standard, the methoxy-trimethyl-phenyl analog of vitamin A, 15,15-dimethyl-retinol and retinyl propionate have been employed. The first two compounds are preferable to retinyl acetate as a marker in having solubility properties more similar to retinol and in not being convertible to retinol by hydrolysis, but they both suffer from not being readily available commercially.

### *Reproducibility*

Type	Procedure 1 Within-day	Procedure 2 Successive days
N	8	10
$\bar{x} \pm SD$	$58.9 \pm 1.5 \mu\text{g/ml}$	$31.6 \pm 1.4 \mu\text{g/ml}$
C.V.	2.5%	4.4%

### *Sensitivity*

The lower detection limit, using 100  $\mu\text{l}$  plasma, is 5  $\mu\text{g}$  retinol/dl for procedure 1 and about the same for procedure 2, depending on the method of detection.

### **Effect of Sample Storage**

Plasma samples kept in the frozen state at  $-20^{\circ}\text{C}$  give the same values for at least two months.

### **Variations and Modifications**

Many methods for the separation and analysis of plasma retinol by HPLC columns have been published in the past few years. Methanol-chloroform (Bligh-Dyer) extraction has been successfully used, saponification can precede analysis to yield total plasma vitamin A values, and different elution solvents and columns may be used. Most procedures use the reversed-phase technique, however, which allows retinol to be eluted as a sharp peak in a few minutes.

### **Comments and Precautions**

The HPLC technique has greatly improved the accuracy and rapidity of plasma retinol analysis while retaining high sensitivity. Thus, it is the method of choice when the instrument is available. The cost of analysis by this procedure is not trivial, however, since prepacked columns are expensive (approximately \$200 US in 1982), instruments are costly (\$6,000-\$30,000 US in 1982) and maintenance problems do arise. The use of a guard column is also advisable to protect the main columns from clogging. When normal phase columns are used, the elution solvent should be equilibrated with the column for at least a day before use.

Needless to say, many peaks are sensed at 280 nm, and a significant number at 325 nm as well. Thus, slight changes in the eluting solvent may be necessary to separate retinol and the internal standard clearly from other absorbing compounds. A UV-absorbing contaminant derived from the rubber seals in vacutainers, which elutes just before retinol in procedure 2, can also interfere with the retinol peak.

The carotenoid phytofluene, which absorbs at 331, 348 and 367 nm in methanol or hexane and fluoresces like vitamin A, is eluted before retinol on the normal phase column (procedure 1) and after it on reversed-phase columns (procedure 2). Beta-carotene and other hydrocarbon carotenoids, which react positively in colorimetric methods, run like phytofluene under the specified assay conditions.

Analysis without use of an internal standard (i.e., by reference to a standard curve

based on retinol or retinyl acetate) is also feasible. Much care must be taken, however, to optimize the extraction of retinol, to minimize losses due to oxidation, and to control the volumes of lipid extracts and aliquots used for HPLC. Thus, unless the overall efficiency of the analysis is shown to be consistently high with test samples beforehand, an internal standard should be employed.

## **Spectrophotometric Method by Ultraviolet Inactivation**

### **References**

1. Bessey, O. A., Lowry, O. H., Brooks, M. J., and Lopez, J. A. (1946) The determination of vitamin A and carotene in small quantities of blood serum. *J. Biol. Chem.* 166:177.
2. Araujo, C. R. C. and Flores, H. (1978) Improved spectrophotometric vitamin A assay. *Clin. Chem.* 24:386.
3. Arroyave, G. and de Funes, C. (1974) Enriquecimiento de azucar con vitamina A. Metodo para la determinacion cuantitativa de retinol en azucar blanca de mesa. *Arch. Latinoamer. Nutr.* 24:147.
4. Utley, M. H., Brodovsky, E. R., and Pearson, W. N. Hemolysis and reagent purity as factors causing erratic results in the estimation of vitamin A and carotene in serum by the Bessey-Lowry method. *J. Nutr.* 66:205.

### **Principle**

Retinol is destroyed when exposed to ultraviolet light. After saponification of a serum sample with alcoholic KOH, retinol and carotenoids are extracted by solvent partition using a mixture of xylene-kerosene. The optical density of the sample extract is read at 460 nm for the determination of "total carotenoids" and at 328 nm for the determination of retinol. The sample extract is then irradiated with ultraviolet light to destroy retinol and its absorbance is read again at 328 nm. The difference in optical density at 328 nm before and after irradiation of the sample is proportional to the concentration of retinol. The concentration of carotenoids and retinol are calculated based on an absorbance factor related to their respective extinction coefficients in the solvent mixture.

### **Apparatus**

Ultraviolet-visible spectrophotometer with appropriate cuvettes.

When the amount of sample to be read is equal to or less than 1 ml, use of a spectrophotometer with a microcell adaptor is essential and black-masked cuvettes are very highly recommended.

Thermoregulated water bath

Vortex mixer

Centrifuge (preferably refrigerated)

Appropriate test tubes and glassware

Automatic pipettes (Eppendorf or Oxford type)

A weak, ultraviolet light source (about 350-390 nm)

Recommended: Black-light tubes General Electric F 20 T 12/BLB 20 watts or Eye FL 20 SBLB. If other lamps are used, their effectiveness in destroying vitamin A must be tested.

Cleanliness is a critical factor in this type of analysis. Consequently, all glassware, after regular washing, should be rinsed with a 50% solution of nitric acid, rinsed again

with sufficient distilled water to remove all traces of nitric acid, and then dried in an oven. The microcells are first washed with a 1:1 mixture of 3N HCl-ethanol, rinsed again with ethanol and finally with the xylene-kerosene mixture. Other cleaning procedures which give similar results may also be used.

## Reagents

Absolute ethanol

KOH, 11 N

Prepared periodically from KOH pellets and stored in a polyethylene bottle.

Alcoholic KOH, 1N

Prepared just before use by mixing 11 N KOH and absolute ethanol in a ratio of 1:10. The left-over alcoholic KOH is discarded.

Xylene (certified A.C.S. Xylenes, Fisher Scientific Company)

Additional purification by distillation is necessary as follows: Distill at around 130-133°C using all glass distillation equipment. Discard approximately 100 ml of the initial and final fractions. The distilled xylene should read less than 0.025 O.D. at 328 nm against water. Store in a dark bottle.

Kerosene (odorless kerosene, Fisher Scientific Company)

It must be additionally purified in the laboratory as follows: Distill at around 193-198°C using all glass distillation equipment. Discard respectively, as in the case of xylene, 100 ml of the initial and final fractions. Purify further with Norite and then filter by twice passing the kerosene-Norite mixture through a double layer of Whatman No. 1 filter paper. The kerosene purified in this fashion should read less than 0.02 O.D. at 328 nm against water. Store in a dark bottle.

Kerosene-xylene mixture

Mix equal volumes of purified kerosene and xylene just before use.

Glycerol, reagent grade (only for the analysis of vitamin A in liver)

Chloroform or ethanol (used only for vitamin A standard)

Vitamin A standard. The USP reference standard for vitamin A is suitable (Chapter III).

## Procedure

### *Preparation of Standard and Determination of the Method-Specific Absorbancy Factor*

The standard is used only for checking the assay conditions; its absorbance is not used in calculating the retinol concentration.

Weigh out approximately 30 mg of the oily USP reference standard solution of vitamin A, which contains approximately 34.4 mg retinyl acetate/g, and dissolve in 100 ml ethanol. The solubility of the oil in ethanol does not permit more concentrated solutions. This solution can be used as a stock if kept in a dark bottle and refrigerated. If a calibration curve is desired, prepare suitable dilutions of the stock standard in ethanol. Carefully determine the absorbance of these solutions at 328 nm before ( $A_0$ ) and after ( $A_1$ ) irradiation with ultraviolet light. Determine the time required for  $A_1$  to reach a plateau under the assay conditions. Calculate net absorbancy values ( $A_0 - A_1$ ) of retinyl acetate solutions for each dilution used under conditions of optimal bleaching. Under proper conditions of irradiation,  $A_1$  should be 3% or less of  $A_0$ . Plot the values with  $A_0 - A_1$  on the y axis and relative vitamin A concentration on the x axis curve. Determine the method-specific absorbancy factor as a function of the retinyl acetate concentration. For the latter, an  $E_{1\text{cm}}^{1\%}$  value at 328 nm of 1565 (Table 2) for retinyl acetate in ethanol is used. To obtain the concentration of the retinyl acetate solution in terms of retinol, an  $E_{1\text{cm}}^{1\%}$  value of 1795 in ethanol is used. This value corrects for the 3% decrease in the  $E_{1\text{cm}}^{1\%}$  value of retinyl esters in ethanol (Table 2).

### *Preparatory Treatment of Blood, Liver or Milk Specimens*

Although the original method was described for the analysis of blood serum, it can be equally applied to milk and liver specimens. Aliquots of carefully homogenized human milk are treated and analyzed as for serum. In the case of liver, a homogenate is first prepared in a 1:1 mixture of distilled water and glycerol. The homogenate should be diluted so that its concentration of retinol falls within a range appropriate for reading in the spectrophotometer.

### *Analytical Steps*

In  $10 \times 75$  mm test tubes place duplicate  $200 \mu\text{l}$  aliquots of distilled water for blanks, a control serum and unknown samples. Add  $200 \mu\text{l}$  of alcoholic KOH to all tubes including blanks. Mix the contents vigorously on a vortex mixer for 10-20 seconds and stopper. Place the tubes in a water bath at  $55^\circ$ - $60^\circ\text{C}$  for 20 minutes. Temperatures above  $60^\circ\text{C}$  should be avoided inasmuch as extracts may become turbid at elevated temperatures.

Prepare a 1:1 mixture of xylene-kerosene while the samples are saponifying in the water bath. At the end of 20 minutes, cool the samples to room temperature and add  $200 \mu\text{l}$  of the xylene-kerosene mixture. Extract retinol by vigorous mixing of each tube on the vortex mixer for at least 30 seconds. Centrifuge the mixture for 5 minutes at  $600$ - $1000 \times g$ . Withdraw the xylene-kerosene layer carefully by use of a constriction micropipette connected to a rubber tube (for mouth suction) or of a Pasteur pipette, and place the sample extract directly into the spectrophotometer cuvettes. Read the optical absorbance at 328 nm for retinol and at 460 nm for total carotenoids. Avoid contamination of the upper organic layer with the aqueous phase, since remixing may cause turbidity.

Transfer the sample extract from the cuvette to glass tubes ( $10 \times 75$  mm) for irradiation. Irradiate all the samples and blanks for 35 minutes using an ultraviolet irradiation source ("black light unit"). As mentioned earlier, the time required for extensive bleaching may vary, depending on the light source used and other assay conditions. In one test laboratory, 60-90 minutes of irradiation were needed. Transfer the irradiated sample extract to the cuvettes and again read their optical absorbance at 328 nm.

### *Calculations*

By use of a method-specific absorbance factor\* (related to the  $E_{1,1\text{cm}}^{1\%}$  value) for retinol of 1688 at 328 nm in the xylene-kerosene mixture, the serum retinol concentration in  $\mu\text{g}/\text{dl}$  can be calculated as follows:

$$\text{Retinol } (\mu\text{g}/\text{dl}) = [A_0 - A_1] \times F_1$$

Where:  $A_0$  = initial optical absorbance reading at 328 nm;

$A_1$  = optical absorbance at 328 nm after ultraviolet irradiation; and

$F_1$  = a proportionality factor, e.g., 592 in the present case.

The method-specific absorbance factor and proportionality factor (F) used by Bessey *et al.*<sup>1</sup> were 1570 and 637, respectively. The F value recommended in this manual (592) is 0.93 of the value given in the Bessey study. The major reason for the difference is the different  $E_{1,1\text{cm}}^{1\%}$  values used for pure vitamin A, 1850 for retinol in hexane in the present manual and 1720 for retinyl palmitate, expressed as free retinol, in ethanol in Bessey *et al.* In that study, no correction was made for the reduced absorption of retinyl esters in ethanol relative to hexane. For most practical purposes, this 7% increment is of little importance.

\*The method-specific absorbance factor for retinol at 328 nm in the xylene-kerosene mixture (1688) is calculated by multiplying the following factors:  $E_{1,1\text{cm}}^{1\%}$  of retinol in hexane at 325 nm (1850), the relative absorbance of retinol in the xylene-kerosene mixture at 328 nm and in hexane at 325 nm (0.96), the bleaching efficiency at 328 nm (0.97) and the saponification yield (0.98).

When exact comparisons are made between studies using the factor of 637 and those using the factor of 592, however, a 7% correction must be made. Similarly, by use of a method-specific absorbance factor (related to the  $E_{1\text{cm}}^{1\%}$  value) for beta-carotene of 2080 at 460 nm in xylene-kerosene, the total carotenoid concentration in serum expressed in terms of beta-carotene is as follows:

$$\text{Total Carotenoids } (\mu\text{g/dl}) = A_{460} \times F_2$$

Where:  $A_{460}$  = absorbance at 460 nm; and

$F_2$  = a proportionality factor, e.g., 480 in the present case.

The interpretation of plasma carotenoid values is discussed in Chapter VII.

### *Reproducibility*

When the method is properly conducted, the coefficient of variation (C.V.) of retinol values for within-run replicates is <5%. The variability found at two levels of serum retinol is shown below:

#### **Within-run variability of serum retinol ( $\mu\text{g/dl}$ )**

Sample No.	N	$\bar{x}$	S.D.	C.V. (%)
1	30	27.5	1.1	4.0
2	24	63.8	1.2	1.9

The coefficient of variation between serum analysis performed on different days has been from 2.3% for samples with "high" retinol concentrations to 5.9% for samples with low retinol levels. Typical examples of between-run variability for different retinol concentrations are illustrated below:

#### **Between-run variability of serum retinol ( $\mu\text{g/dl}$ )**

Sample No.	N	$\bar{x}$	S.D.	C.V. (%)
1	10	67.6	1.6	2.4
2	18	33.6	1.3	3.9
3	14	20.2	1.2	5.9
4	5	13.6	0.8	5.9

Thus, when this method is used, the quality control goal should be to restrict the average coefficient of variation for a group of samples to 5% or less.

### *Sensitivity*

Since the determination of vitamin A and carotenoids by this method is based on the  $E_{1\text{cm}}^{1\%}$  values of the substances, the sensitivity will depend on the extraction efficiency, the length of the light path, and the characteristics of the spectrophotometer used. Under the conditions described here, retinol levels down to 5  $\mu\text{g/dl}$  can be measured accurately. Lower retinol levels should be reported as <5  $\mu\text{g/dl}$ .

### **Effect of Sample Storage**

Samples may be stored at  $-20^\circ\text{C}$  for several months without affecting the vitamin A levels. After four-and-a-half months of storage at  $-20^\circ\text{C}$ , no change occurs in serum retinol values. To maximize stability, storage under nitrogen is strongly recommended.

Human milk is equally stable. The vitamin A content of liver is stable at room temperature up to 26 hours after removal of the organ, and indefinitely if stored properly at  $-20^{\circ}\text{C}$  or lower. In stored samples, vitamin A is more stable than carotenoids.

## Variations and Modifications

- The size of the serum sample can be changed as desired from  $200\ \mu\text{l}$  up to 1.5 or 2.0 ml. This modification, of course, will depend on sample volume availability. A large sample volume may facilitate the analytical procedure by avoiding the use of specialized glassware, micro-cell adaptors in the spectrophotometer and the meticulous, and sometimes difficult, handling of small amounts of sample extracts.

- Although the ratio of sample volume to the volume of alcoholic KOH added for saponification must always be 1:1, the ratio of sample size to the volume of solvent added for the extraction procedure can be modified. Instead of using a 1:1 ratio, larger volumes of solvent (xylene-kerosene) can be used. This modification may facilitate the extraction procedure and will provide a larger volume of sample extracts that are easier to handle. Keep in mind, however, that the more diluted the vitamin A content of the sample becomes, the lower the optical density (O.D.) readings will be. This may be a critical factor to consider when dealing with samples with low vitamin A levels. If the proportion of sample volume to the volume of the solvent added is changed from a 1:1 ratio, make sure that proper correction is made for the dilution of the sample when doing the calculations.

- Due to its high boiling point, the xylene-kerosene mixture is the preferred solvent. Only kerosene with an initial O.D. below 2 is suitable. If odorless kerosene is not available, suitably purified jet fuel (Turbo-fuel A-1), which is available at major airports, may be substituted. About 300 ml of the solvent are distilled in 15 ml fractions. Fractions with an O.D.  $<0.5$  at 328 nm are pooled and used as a substitute for kerosene. If other organic solvents are employed, appropriate  $E_{1\%}^{1\text{cm}}$  values must be selected. The volatility of cyclohexane, which is otherwise excellent, reduces its utility. This constraint is critical when small sample volumes are used.

## Comments and Precautions

The spectrophotometer cuvettes must be carefully matched since one is usually dealing with rather low readings.

- The position of the cuvettes and the optical geometry of the spectrophotometer must be checked very carefully, especially in instruments not equipped with an automatic cuvette positioner. It is also advisable to check periodically the accuracy of the wavelength setting.

- When the environmental temperature and humidity are high, all analytical procedures should be performed in an air-conditioned room.

- The irradiation condition must be established initially and checked periodically to ensure that the absorbance of an alcoholic solution of retinyl acetate falls to a constant value close to 0 upon exposure to the UV source. This is usually accomplished after 10-15 minutes of exposure. Irradiation for 30 minutes usually gives a sufficient security

margin. With weaker light sources or more UV-absorbing glass tubes, irradiation times of 60-90 minutes may be necessary.

- Over-irradiation of serum extracts, on the other hand, may either destroy other absorbing compounds in the extract or produce absorbing products.
- Since some types of glass tubes are opaque to ultraviolet light, irradiation curves must be run each time tubes are changed. Another source of error is the decrease in intensity of the irradiation source a short time before burning off. Be sure to replace the source periodically.
- Poor quality rubber stoppers may contaminate the sample, especially when in contact with KOH. The quality of rubber in vacutainer stoppers has proved satisfactory, but direct contact with the reagents and the materials under assay should still be avoided.
- A chart-recorder may be used to improve the speed and ease of the method.
- As described, this method measures total vitamin A and does not differentiate retinol from retinyl esters.

## Colorimetry Using Trifluoroacetic Acid (TFA)

### References

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### Principle

The proteins of plasma or serum are precipitated with ethanol, and the vitamin A and carotene extracted into hexane (or petroleum ether). The carotene concentration is determined by measuring the absorption of the extract at 450 nm. Following evaporation of the solvent, vitamin A dissolved in chloroform is determined by reading the intensity of the blue color developed after addition of the trifluoroacetic acid-chloroform reagent at two time points. A correction is made for the concentration of total carotenoids, since carotenoids, when present in high amounts, contribute to the intensity of blue color.

When the serum level of vitamin A is low and the carotenoid concentration is high, falsely low vitamin A values can be avoided by first removing carotenoids by chromatography on alumina columns (pps. 36-38).

## Apparatus

Colorimeter or spectrophotometer with appropriate cuvettes, adapted to take microcuvettes for the microprocedure

Vortex mixer

Glass stoppered test tubes

Pipettes, 0.1, 2.0, 3.0 ml (or 0.01, 0.05, 0.075 and 1.0 ml for microprocedure) (Eppendorf or equivalent automatic pipettors recommended).

Centrifuge, desk top

Water bath, 40-60°C constant temperature

Nitrogen (or other inert gas)

Volumetric flasks, 10 ml and 100 ml

## Reagents

Ethanol, 95% or absolute

Hexane (or petroleum ether, bp 38-57°C)

Chloroform, reagent grade

Acetic anhydride, reagent grade

Trifluoroacetic acid, reagent grade (trichloroacetic acid can be substituted)

A 50% solution of TFA in chloroform (v/v) is prepared just prior to use.

Vitamin A standard, USP reference capsule of all-*trans* retinyl acetate in oil or purified crystalline retinol or retinyl acetate.

Beta-carotene standard, all-*trans*.

Use only freshly opened ampules that have been sealed under nitrogen. Carotene is highly unstable; even unopened standards sometimes have deteriorated.

## Procedure

### Macroprocedure

All procedures should be carried out in dim light and caution exercised to avoid excessive exposure to oxidation.

Duplicate 2 ml aliquots of serum or plasma are pipetted into glass stoppered test tubes. An equal volume (2 ml) of ethanol is added dropwise with mixing to give a 50% solution (v/v). At this concentration the protein-retinol bond is disrupted, the protein precipitated and free retinol and retinyl esters are available for extraction by addition of 3 ml hexane (or petroleum ether). The tube is stoppered and the contents mixed *vigorously* on the vortex mixer for 2 minutes to insure complete extraction of carotene and vitamin A; then centrifuged for 5-10 minutes at 600-1000 × g to obtain a clean separation of phases. Two ml of the upper hexane (or petroleum ether) extract is pipetted into cuvettes and the cuvettes are capped. Absorbance due to carotenoids at 450 nm is read against a hexane (or petroleum ether) blank ( $A_{450}$ ).

After determining  $A_{450}$  the cuvettes are removed and the hexane (or petroleum ether) is evaporated just to dryness under a gentle stream of nitrogen in a 40-60°C water bath while avoiding splashing on the test tube wall. If evaporation cannot be carried out in the cuvettes, transfer to another tube with rinsing, and proceed. Just at the point of dryness, the residue is immediately redissolved and dehydrated by addition of 0.1 ml of a mixture

of chloroform-acetic anhydride (1:1 v/v). The cuvettes or tubes should be capped to minimize evaporation and should be protected from light.

The spectrophotometer at 620 nm is preset to zero absorbance with a blank consisting of 0.1 ml chloroform-acetic anhydride mixture and 1.0 ml TFA-chloroform chromagen reagent.

The cuvette containing the sample is placed in the spectrophotometer and 1.0 ml TFA chromagen reagent added to the cuvette from a rapid delivery pipette. Alternatively, the reagent can be added to the extract in a separate tube and rapidly transferred to the cuvette. These steps must be carried out rapidly and with care since the blue color fades quickly and the chromagen reagent is highly corrosive. Record the absorbance reading ( $A_{620}$ ) at exactly 15 seconds ( $t_{15}$ ) and at 30 seconds ( $t_{30}$ ) after addition of the reagent.

### *Microprocedure*

The microprocedure is essentially the same as the macroprocedure, but is adapted to a smaller scale. Accurate results depend on great care in pipetting and transferring small volumes, and minimizing possible losses from evaporation, oxidation and light exposure.

A volume of 100  $\mu$ l of serum or plasma is pipetted into 6  $\times$  50 mm glass stoppered test tubes. An equal volume of ethanol is added with mixing followed by 150  $\mu$ l of hexane or petroleum ether (light petroleum)\* (1:1:1.5 v/v/v ratio serum:alcohol:solvent, respectively). If a smaller (50  $\mu$ l) or larger (200  $\mu$ l) volume of serum is used, the volume of other reagents is adjusted proportionately.

The tubes are immediately stoppered and vigorously vortexed for 2 minutes to extract carotenoids and vitamin A, and then are centrifuged for 5-10 minutes at 3000  $\times$  g to achieve a clean phase separation. Thereafter 100  $\mu$ l hexane (petroleum ether) extract is transferred to a microcuvette by means of a micropipette, and the absorbance due to carotenoids at 450 nm is read against a hexane (or petroleum ether) blank.

The spectrophotometer, or preferably a spectrophotometer of quality equivalent to a Beckman DU, is set at 620 nm and then zeroed against a reagent blank containing 10  $\mu$ l chloroform-acetic anhydride reagent and 100  $\mu$ l freshly prepared TFA-chloroform chromagen reagent.

The sample is then transferred from the microcuvette to a clean 6  $\times$  50 mm test tube, the cuvette rinsed once with 50  $\mu$ l hexane (or petroleum ether), and the rinse solution added to the sample in the test tube. The extract is evaporated just to dryness under a gentle stream of nitrogen to avoid splashing in a 40-60°C water bath. The residue is redissolved in 10  $\mu$ l chloroform-acetic anhydride (1:1 v/v) reagent. Then 100  $\mu$ l freshly prepared TFA-chloroform chromagen reagent is added from a rapid delivery pipette and with vigorous mixing. The solution is rapidly transferred to the microcuvette by means of a microtransfer pipette. A reading at  $A_{620}$  is obtained against a TFA reagent blank at exactly 15 seconds ( $t_{15}$ ) and again at 30 seconds ( $t_{30}$ ) after addition of the chromagen. Careful timing is essential since the color fades rapidly.

### *Standardization*

*Beta-carotene  $A_{450}$  calibration factor.* A stock carotene solution is prepared by weighing exactly 50 mg freshly opened all-*trans* beta-carotene standard and dissolving it in a few milliliters of chloroform. Bring the solution to exactly 100 ml in a volumetric

\*Light petroleum (40-60 degrees bp), commonly called petroleum ether, is the light fraction of short chain hydrocarbons (e.g., pentane, hexane) distilled at the temperature specified.

flask with hexane (or petroleum ether). Prepare just prior to use, since the solution deteriorates on storage. This is the *stock* beta-carotene solution containing 0.5 mg/ml. Protect from light.

An *intermediate* standard containing 5  $\mu\text{g/ml}$  is prepared by diluting 1 ml stock carotene solution to 100 ml in a volumetric flask with hexane (or petroleum ether). This solution is stable only for a few hours and should be made just prior to use.

*Working standards* are prepared from the intermediate standard by diluting with hexane (or petroleum ether) in each of four 10 ml volumetric flasks 1, 2, 4, and 8 ml of intermediate standard solution. This results in solutions containing 0.5, 1.0, 2.0 and 4.0  $\mu\text{g/ml}$  of beta-carotene, respectively.

Fill the cuvette with the carotene working standards and read  $A_{450}$  against a hexane (or petroleum ether) blank. Plot a standard curve and from it determine the factor for carotene (FC) where:

$$FC_{450} = \frac{\mu\text{g carotene/ml}}{A_{450}}$$

*Beta-carotene  $A_{620}$  correction factor.* Carotenoids also react with the TFA-chloroform chromagen to yield a blue color at  $A_{620}$ . Therefore, it is necessary to run a chromagen beta-carotene standard curve in order to calculate a correction factor in obtaining vitamin A values. This correction is not necessary if serum (plasma) contains carotene in concentrations under 50  $\mu\text{g/dl}$ , as the contribution to the blue color in this concentration range is negligible. It must be emphasized that this is *not* an assay for beta-carotene; this measurement is only useful in correcting vitamin A values.

Carotene standards in chloroform are prepared to contain 4.0, 8.0 and 10.0  $\mu\text{g/ml}$ . Pipette duplicate 0.1 ml aliquots of each standard solution into cuvettes or suitable small tubes. Rapidly add 1.0 ml TFA-chloroform chromagen and mix vigorously. Transfer to the cuvette and read absorbance at 620 nm at 15 seconds ( $t_{15}$ ) and 30 seconds ( $t_{30}$ ) exactly as described previously. The  $t_{15}$  and  $t_{30}$  values are plotted on rectangular coordinate graph paper with the ordinate containing  $A_{620}$  values and the abscissa containing time after addition of chromagen. Extrapolate to  $t_0$  with a ruler to determine the  $A_{620}$  at  $t_0$ . Note that the decay in absorbance is linear for at least 30 seconds. The  $t_0$  absorbance value, therefore, can be determined by the formula:  $A_{t_0} = A_{t_{15}} + (A_{t_{15}} - A_{t_{30}})$ . Determine a factor ( $FC_{620}$ ) where:

$$FC_{620} = \frac{\mu\text{g carotene/ml}}{A_{620}}$$

In measuring vitamin A, the absorbancy correction at 620 nm for carotenoids is:

$$\frac{2 \times A_{450} \times FC_{450}}{FC_{620}}, \text{ in which the factor of 2 derives from the difference in the dilution of the carotenoids and vitamin A in their respective assays.}$$

*Vitamin A standard curve.* Retinyl acetate or retinol can be used as standards for preparation of reference curves since both have identical blue color characteristics in the analytic procedure after making appropriate molecular weight adjustments to convert

retinyl acetate (MW=328) to retinol (MW=286) equivalents (i.e., when the acetate is used  $286/328 = 0.872$ ). Any of the standards noted in Chapter III can be used. The USP reference capsule of retinyl acetate in oil, for example, is suitable. It is said to contain about 34.4 mg all-*trans* retinyl acetate/g solution, but it is necessary periodically to check the concentration of dilutions by spectrophotometry. Standards should be kept refrigerated and protected from light. They should not be used beyond storage of two days without redetermining the concentration spectrophotometrically.

A *stock* vitamin A standard containing approximately 50-60  $\mu\text{g}/\text{ml}$  is prepared by carefully weighing an appropriate amount of the vitamin A standard and diluting with hexane in a volumetric flask. The exact concentration in  $\mu\text{g}$  retinol/ml is obtained by determining the absorbance at 325 nm and by using the  $E_{1\%}^{1\text{cm}}$  value of 1850 for retinol.

Working standards are prepared in 10 ml volumetric flasks by diluting the appropriate volume of the stock solution with hexane (or petroleum ether) to obtain concentrations in the range of 6, 12, 24, 36 and 60  $\mu\text{g}/\text{ml}$ .

Then 0.1 ml of each working standard is pipetted into cuvettes for reactions with 1.0 ml TFA-chloroform chromagen exactly as previously described, reading  $A_{620}$  at 15 seconds ( $t_{15}$ ) and 30 seconds ( $t_{30}$ ).

The  $A_{620}$  values for  $t_{15}$  and  $t_{30}$  are plotted on a graph where the ordinate is the  $A_{620}$  values and the abscissa is the time after addition of chromagen. Using a ruler, extrapolate to  $t_0$  to obtain  $A_{620}$  for each working standard. This is the theoretical time of maximum color intensity obtainable since the decay in blue color is linear for at least 30 seconds after chromagen addition. Alternatively,  $t_0$  absorbance readings can be determined as described above.

A standard curve is plotted from the  $A_{620}$  values at  $t_0$  on ordinary rectangular coordinate paper where the ordinate is the  $A_{620}$  value and the abscissa is the  $\mu\text{g}$  vitamin A/tube. From the curve calculate a factor ( $FA_{620}$ ) where:

$$FA_{620} = \frac{\mu\text{g vitamin A/tube}}{A_{620}}$$

### Calculations

Based on the procedure outlined above using 2 ml of serum (plasma) extracted into 3 ml solvent, serum values for carotenoids and vitamin A can be calculated by the following formulas:

$$\text{Total carotenoids (as } \mu\text{g beta-carotene/dl)} = A_{450} \times FC_{450} \times 150$$

where  $FC_{450}$  is the constant determined in each laboratory and 150 accounts for dilution factors;

$$\text{Vitamin A (as } \mu\text{g retinol/dl)} = [A_{620} - 2 \times A_{450} \times FC_{450}] \times FA_{620} \times 75$$

If the relative volumes of serum and reagents used in the micro procedure are modified, the calculation must reflect these changes.

### Reproducibility

The coefficient of variation (C.V.) observed at two different levels of plasma vitamin A concentrations is:

	Within-day	Between-days
Mean $\pm$ S.D.	61.5 $\pm$ 0.4 $\mu\text{g}/\text{dl}$	62.9 $\pm$ 2.2 $\mu\text{g}/\text{dl}$
C.V.	0.6%	3.5%
N	16	20
Mean $\pm$ S.D.	29.9 $\pm$ 0.5 $\mu\text{g}/\text{dl}$	32.4 $\pm$ 2.5 $\mu\text{g}/\text{dl}$
C.V.	1.8%	7.8%
N	16	20

Clearly, the coefficient of variation is very satisfactory for within-day analyses and acceptable, even at lower mean vitamin A values, for studies conducted on different days.

### *Sensitivity*

The lower detection limit is about 9  $\mu\text{g}$  retinol/dl. This detection limit is somewhat higher than that using HPLC or UV bleaching because a transient color is being measured. Under assay conditions absorbancy values  $<0.02$  units are poorly reproducible.

### *Comparison of Methods Involving UV Bleaching and TFA Colorimetry*

In a test laboratory the above two methods were carefully compared with identical blood samples under several conditions. The results are presented below:

Sample	N	Ratio $\left(\frac{\text{CM}}{\text{UB}}\right)^*$	r
Immediate analysis	9	1.03 $\pm$ 0.08	0.95
Sera frozen under N <sub>2</sub>	16	1.03 $\pm$ 0.08	0.98

\*The mean of the ratios of values obtained by the colorimetric method (CM) to that obtained by ultraviolet bleaching (UB)  $\pm$  S.D.

Thus, under ideal conditions of sample handling, the agreement between these methods was excellent. When blood was kept for six or 12 hours at 0°C in the dark before centrifugation, however, the ratio increased (1.24  $\pm$  0.52) and the correlation coefficient fell ( $r=0.76$ ). The ratio for sera kept frozen under air for a significant period was high (4.26  $\pm$  1.13) with a very poor correlation. Thus the importance of maintaining an anaerobic state in stored serum values, particularly when the colorimetric method is used, is stressed by this comparative study.

### **Effect of Storage**

Following collection of blood, serum or plasma should be separated as early as possible and certainly within 24 hours. Preferably, the analysis should be carried out immediately. If this is not possible, serum or plasma should be frozen at  $-20^\circ\text{C}$  until analyzed. The samples should be placed in tubes that allow a minimum air head space, tightly stoppered and protected from light. Stability is much increased by flushing the tubes with nitrogen or some other inert gas prior to sealing. Storage without thawing results in little or no loss up to about one month and approximately a 10% loss over a four-month storage period. Storage in a freezer has been reported not to affect values when the trifluoroacetic acid chromagen is used<sup>1</sup>, but to induce spurious high values when antimony trichloride is used<sup>2</sup>. Each laboratory should determine the stability of standardized samples under its storage conditions.

## Variations and Modifications

- TFA is the chromagen of choice because it is less sensitive to moisture than the antimony trichloride chromagen originally proposed. Trichloroacetic acid also has been used to replace TFA with acceptable results (pps. 56-59).

- 1,2 Dichloroethane has been used to replace chloroform in preparation of the chromagen by adding one part TFA to three parts 1,2 dichloroethane. The dichloroethane is also used to replace chloroform in redissolving the residue after evaporation of hexane from the sample extract. In this procedure the color is read at 616 nm 10 seconds after addition of the chromagen (H. Sauberlich, personal communication).

## Comments and Precautions

- Maximum blue color intensity develops at a TFA concentration of at least 40% in the final mixture. Adjustments that are made in reagent or sample volumes must be such that at least a 40% final TFA concentration is maintained.

- The decay rate of blue color may vary for standards and unfractionated serum (plasma) extracts, but in both cases the decay is linear at least up to 30 seconds. Greatest accuracy is achieved by extrapolation of absorbance readings at  $t_1$  and  $t_2$  to  $t_0$ , where theoretically the maximum color intensity is achieved. Standard curves should also be based on the  $t_1$  readings.

- Working standards should be selected in the same concentration range as the samples to be tested.

- It is necessary to check the concentration of vitamin A in the reference standards periodically by spectrophotometry.

- The TFA reagent is highly corrosive and maximum caution should be used to avoid contact with skin or mucous membranes. Never pipette either TFA or chloroform by mouth.

- If vitamin A values are below 20  $\mu\text{g}/\text{dl}$  and the correction for the formation of the carotenoid chromophore at 620 nm is  $>20\%$  of the vitamin A values, the calculated vitamin A values may be significantly in error. In such cases, preliminary removal of carotenoid contaminants by chromatography on approximately 6% water-deactivated alumina columns is advised. The columns are prepared and the retinol eluted as described on page 37.

- Slight hemolysis does not interfere with the reaction and no significant change occurs on storage, provided that samples are not repeatedly thawed and refrozen. These points should be checked, however, in each laboratory under the conditions used.

- Any plasma volume that contains sufficient vitamin A to be measured by the procedure can be selected and the reagent volumes adjusted accordingly.

## CHAPTER V

# Other Methods

## Fluorescence Assay

### References

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### Principle

Plasma (200  $\mu$ l) is extracted with ethanol and hexane, and the fluorescence readings at 480 nm when the sample is excited both at 330 nm and at 365 nm are recorded. On the basis of fluorescence values obtained with standard retinol and purified phytofluene, the major fluorescent contaminant found in plasma, the concentration of vitamin A can be calculated by a correction formula.

### Apparatus

Ultraviolet-visible spectrophotometer  
Fluorescence spectrophotometer  
Centrifuge  
Hand ultraviolet lamp (350 nm maximum excitation)  
Water bath  
Vortex mixer

Chromatography columns---one 10 mm i.d. with drawn tip and one 20 mm i.d.  
 Magnetic stirrer  
 Flash evaporator  
 Stoppered centrifuge tubes, 15 ml capacity  
 Domestic food blender

## Reagents

All-*trans* retinyl acetate, USP vitamin A reference standard capsules

All-*trans* retinol

Alumina for column chromatography, neutral and basic

Hexane, glass distilled

KOH pellets, reagent grade

Absolute ethanol, reagent grade

Ethanol, 95%

Distill from KOH pellets (ca. 50 g/liter)

Quinine sulphate USP

Prepare a 1% solution of quinine (10 mg/ml) by dissolving 1.05 g of the salt in 100 ml 0.1 N H<sub>2</sub>SO<sub>4</sub>. By sequential serial dilution (1/100 × 1/100 × 1/10) prepare a quinine solution containing 0.1 μg/ml. Avoid contaminating the H<sub>2</sub>SO<sub>4</sub> and the quinine solutions by contact with the skin which will release fluorescent substances. The water used to dilute the acid must be distilled.

Methanol, glass distilled

Fresh tomatoes

Nitrogen (oxygen-free)

## Procedure (Thompson, Erdody and Maxwell, 1973)

### Extraction and Analysis

Dilute serum or plasma (0.2 ml) with 1 ml water and mix with 1.0 ml ethanol in a 15 ml stoppered centrifuge tube. Add 5 ml hexane and blend thoroughly for one minute using a vortex mixer. Stopper the tube and centrifuge for five minutes at 1000 rpm. Keep the tubes stoppered and protect them from light until the hexane extracts can be read in the fluorometer.

Set the slits of the spectrofluorometer to 8 nm, the emission wavelength at 480 nm and the excitation wavelength at 365 nm. Insert the standard quinine solution (0.1 μg/ml), select the correct coarse sensitivity setting (e.g., 3) and adjust the fine sensitivity knob to give a fluorescence reading of 50. Change the excitation wavelength to 330 nm and record the reading (q). The instrument is now standardized. The fine sensitivity controls should not be moved during subsequent readings.

Insert the hexane extract of a plasma sample. Set the coarse sensitivity setting to 30 and read the fluorescence at 480 nm with excitation wavelengths of 330 nm (x) and 365 nm (y).

### Calculation

The vitamin A concentration in plasma, expressed in μg retinol/dl, is calculated from the formula:

$$\text{Retinol} = \left[ \frac{P}{P - A} \left( \frac{Q}{q} x - \beta_2 \right) - \frac{1}{P - A} (y - \beta_1) \right] \frac{2500 S}{R}$$

where:

P is the ratio of the emission intensity of standard *trans*-phytofluene at 480 nm when the excitation wavelengths are 365 and 330 nm, i.e.,  $P_{365}/P_{330}$  or  $P_1/P_2$ ;

A is the ratio of the emission intensity of standard retinol (or saponified retinyl acetate) at 480 nm when the excitation wavelengths are 365 and 330 nm, i.e.,  $R_{365}/R_{330}$  or  $R_1/R_2$ . In measuring P and R values, the fluorescence of hexane is assumed to be negligible. If not, fluorescent corrections should be made before calculating P and A;

Q and q are the emission intensities of the standard quinine solution at 480 nm with excitation at 330 nm at the time of initial standardization and just prior to reading the samples, respectively;

x and y are the emission intensities of the sample at 480 nm with excitation at 330 and 365 nm, respectively;

$\beta_1$  and  $\beta_2$  are the emission intensities of the reagent blank at 365 and 330 nm, respectively;

S is the concentration of the standard retinol solution ( $\sim 0.1 \mu\text{g}/\text{ml}$ );

R is the fluorescence of standard retinol at 330 nm corrected for any attenuation between the standard and the sample.

For example, when the sensitivity settings used for determining the fluorescence at 480 nm of the standard retinol solution is 10 and the sample is 30 when excited at 330 nm, then  $R = R_{330} F$ , where F is the experimentally measured attenuation between settings 30 and 10, usually equal to 3.

2500 is a proportionality factor derived by multiplying the dilution of the plasma sample in transferring retinol to hexane (25-fold) by 100 to provide concentration values in  $\mu\text{g}/\text{dl}$ .

### Determination

Since phytofluene is not available commercially, it must be prepared. The easiest procedure is to chromatograph the hexane fraction of human plasma extracts on deactivated alumina. Usually phytofluene is the major, if not sole, fluorescent polyene. After irradiation to the all-*trans* isomer, this phytofluene fraction, after proper spectrophotometric identification, may be used as the reference standard.

Alternatively, *cis*-phytofluene may be purified from tomatoes. Although tomato paste may be used, fresh tomatoes usually yield a better product. Homogenize fresh tomatoes (400 g) in a domestic blender with 400 ml methanol. Filter the mixture with suction through Whatman No. 1 filter paper and rinse the solid residue twice with 100 ml methanol. The methanol extract is discarded. Stir the red solid residue in 400 ml hexane for 30 minutes using a magnetic stirrer and then filter and evaporate the extract in a flash evaporator. This extract should be protected from light.

Chromatograph the extract on a column of basic alumina (2 cm diameter  $\times$  8 cm) weakened with 2% water. Elute with hexane until the phytofluene moves in front of the colored bands: the phytofluene can be located using a hand ultraviolet lamp very briefly and at a maximum distance. If several fluorescent fractions are present, identify phytofluene by its absorption spectrum ( $\lambda_{\text{max}}$  in hexane of 331 nm, 348 nm (major) and 367 nm). Phytofluene will be eluted completely with 2% diethyl ether in hexane; stop

collecting the fraction when the colored carotenoids reach the bottom of the column. Evaporate the phytofluene fraction to a small volume (but *not* to dryness) using a flash evaporator and, finally, under a stream of nitrogen. This preparation may be stored refrigerated for several months. The preparation predominantly contains *cis*-phytofluene. The fluorescence spectrum can be checked on an aliquot using narrow excitation slits (ca. 2 nm bandpass).

Irradiation of this sample with ultraviolet light will convert the *cis* to the *trans* isomer, with a consequent change in fluorescence intensity up to double its original value. Since *cis*-phytofluene in plasma is also converted to the *trans*-form under the conditions of analysis, *trans*-phytofluene (prepared by gently irradiating natural phytofluene until the fluorescence intensity stabilizes) may also be used as the reference standard.

After standardizing the spectrofluorometer with standard quinine solution as indicated above, the coarse sensitivity knob is moved to 1 and the fluorescence of a suitably diluted *trans*-phytofluene solution (2-8 ng/ml) in hexane is measured at 480 nm with excitation wavelengths of 365 nm ( $P_1$ ) and 330 nm ( $P_2$ ). P is the ratio of  $P_1/P_2$ .

#### *Determination of S and A*

All-*trans* retinol (10 mg) is dissolved in 50 ml absolute ethanol. After a twentyfold dilution of a 1.0 ml aliquot in ethanol, 0.6 ml of the diluted solution (Solution A) (~10  $\mu$ g/ml) is pipetted into a 1 cm cuvette containing 2.4 ml ethanol, the solution is mixed and the absorbancy at 325 nm is determined. By use of an  $E_{1\%}^{1\text{cm}}$  value of 1850, the retinol concentration is calculated. An aliquot of solution A is then appropriately diluted with hexane to yield a reference retinol solution with 0.10  $\mu$ g retinol/ml. Other dilutions can of course be used as long as the exact concentration in the reference solution is known. Retinol may also be prepared by saponifying retinyl acetate.

After standardizing the spectrofluorometer with standard quinine solution as indicated above, the coarse sensitivity knob is moved to 10 and the fluorescence of the reference retinol solution at 480 nm is measured with excitation at 365 nm ( $R_1$ ) and 330 nm ( $R_2$ ). The value of A is the ratio of  $R_1/R_2$ .

#### *Determination of $\beta$ (Blank Correction)*

The extraction procedure is conducted exactly as outlined on page 32 except that 0.2 ml distilled water is used in place of serum. After the instrument is standardized with quinine, fluorescence readings of the blank hexane extract at 480 nm are taken with excitation at 365 nm ( $\beta_1$ ) and 330 nm ( $\beta_2$ ).

#### *Reproducibility*

Within-run	
N	10
$\bar{x} \pm \text{S.D.}$	$65 \pm 1.8$
C.V.	2.8%

#### *Sensitivity*

The sensitivity of the fluorescence assay is very high, at least tenfold greater than methods based on absorption spectra or colorimetric analysis. Under the conditions of analysis a solution containing 10 ng retinol/ml, for example, gives significant fluorescence at 330 nm (e.g., 30 arbitrary units). The main limitation in the use of the fluorescence assay is the presence of fluorescent contaminants, drawn either from the extracted tissue or present in the solvents, rubber or plastics used in the isolation process.

A further complication is that retinyl esters upon irradiation give rise to a highly fluorescent breakdown product.

#### *Comparison With Other Methods or Procedures\**

In comparison with the method described here, prior alumina chromatography or prior solvent partition of the plasma extract did not significantly affect the values obtained. Similarly, the colorimetric assay using antimony trichloride gave values from 0.77 to 1.16 times that given in the present procedure, depending on the exact procedure used. Prior removal of carotenoids can improve the accuracy of the vitamin A assay. Dilution of the original sample directly with ethanol without adding water may improve the extractability of vitamin A.

#### **Storage**

It has been reported that plasma samples can be stored frozen without change of vitamin A values. Because of the sensitivity of the assay and the problem with fluorescent contaminants, however, standard serum samples with known vitamin A content should be stored with each group of frozen experimental samples and analyzed with them (Chapter VI).

#### **Acceptable Variation and Modifications**

Vitamin A in liver,<sup>2</sup> milk,<sup>3</sup> dairy products<sup>4</sup> and foods<sup>5</sup> may be analyzed by fluorometry using the correction formula. In almost all cases saponification must precede hexane extraction and analysis. Before applying the correction formula to foods, however, the presence of fluorescent substances other than phytofluene must first be ascertained. In cases in which phytofluene is absent, such as in most milk samples, the phytofluene correction can be ignored and a simplified formula used, i.e.,  $\mu\text{g retinol/dl} = (\frac{Q_x - \beta_2}{q}) \frac{SD}{R}$  where D is the dilution factor.

Serum phytofluene concentrations can also be determined from the analysis by measuring the fluorescence of a standard *trans*-phytofluene solution ( $E_{480}^{480}$  at 348 nm in hexane of 1632) under assay conditions. The formula used, suitably corrected for vitamin A fluorescence, is as follows.

$$\mu\text{g phytofluene, dl} = \left[ \left( \frac{1}{P-A} \right) (y - \beta_1) - \left( \frac{1}{P-A} \right) \left( \frac{Q_x - \beta_2}{q} \right) \right] \frac{2500 P_s}{F_s}$$

where  $P_s$  is the concentration of phytofluene in the standard solution ( $\mu\text{g/ml}$ ), and  $F_s$  is the fluorescence of the standard phytofluene solution at 480 nm with excitation at 360 nm, suitably corrected for attenuation.

#### **Comments and Precautions**

The concentration of phytofluene in the blood is a function of the dietary intake of fruit and vegetables or their products, whereas the retinol concentration is relatively constant in a given individual under fasting conditions. In some individuals, the phytofluene correction can be as great as the plasma vitamin A concentration; in others it is trivial. The mean phytofluene correction for well-nourished Canadians was found to be  $28.9 \pm 13.1$  (S.D.)  $\mu\text{g retinol}$ , clearly a value that cannot be ignored in routine vitamin A analysis.<sup>6</sup> As might be expected from their different dietary sources and metabolic pathways, phytofluene and retinol concentrations in the plasma are not correlated.

Despite its exquisite sensitivity and apparent ease of analysis, great care must be employed in using this method. The readings of spectrofluorometers often drift, necessitating repeated standardization. Slit openings, both on the excitation and emission monochromators, must be varied to obtain the most stable area of operation while maintaining satisfactory resolution. Contaminating sources of fluorescence (e.g., rubber stoppers or tubing, plastic materials, solvents, and skin secretions) must be rigorously eliminated. Attenuation factors must be determined experimentally. Over-irradiation of samples must be avoided, inasmuch as both destruction of vitamin A and the appearance of new fluorescent substances can occur. In general, the *lowest* sensitivity which allows satisfactory readings should be used in order to minimize the effects of contaminating materials rather than a high sensitivity which necessitates extensive dilution of the sample.

Because of the significant cost of the instrument and the laborious calibration procedure required, fluorescence assay should be reserved for use in large scale surveys. When used properly, however, the fluorescence method is quick, sensitive, accurate, inexpensive and can be automated. But more than most other methods, it requires great care.

## Separation of Retinol and Retinyl Esters

### Group Separation by Alumina Column Chromatography

#### References

1. Thompson, J. N., Erdody, P., Brien, R., and Murray, T. K. (1971) Fluorometric determination of vitamin A in human blood and liver. *Biochem. Med.* 5:67.
2. Vahlquist, A. (1974) A simplified technique for determination of the vitamin A composition in biological fluids. *Internat. J. Vit. Nutr. Res.* 44:375.
3. Awdeh, Z. L. (1965) Separation of vitamin A from carotenoids in micro samples of serum. *Analyt. Biochem.* 10:156.
4. Loerch, J. D. and Underwood, B. A. (In press) Vitamin A determinations in human blood: An analysis of methodology. *Analyt. Chim. Acta.*

#### Principle

The hexane extract of a plasma sample from which proteins are precipitated by ethanol is placed on a water weakened column of alumina. The lipids are eluted by organic solvent mixtures of increasing polarity: non-polar carotenoids first, retinyl esters next, and then retinol. The eluted fractions are then analyzed for vitamin A by spectrophotometry, by colorimetry, or by fluorometry.

#### Apparatus

Spectrophotometer, colorimeter or fluorometer  
10 ml volumetric flasks  
Centrifuge  
Vortex mixer  
Volumetric pipettes, micropipettes  
Long neck funnels or Pasteur pipettes  
Glass wool

## Reagents

Alumina, designated suitable for chromatography  
Hexane, nanograde for subsequent fluorometry  
Ethanol, absolute  
Ethyl ether, peroxide free, reagent grade  
Retinyl ester and retinol standards

## Procedure

### *Preparation of Partially Deactivated Alumina*

Neutral alumina ( $\text{Al}_2\text{O}_3$ ) in fine granular form is heated in an oven at  $120^\circ\text{C}$  overnight and then cooled in a desiccator. It is partially deactivated by adding 4-6% distilled water and shaking. The mixture is allowed to equilibrate for a minimum of two hours before use and preferably overnight.

The amount of water necessary for proper deactivation of alumina will vary from bottle to bottle. To determine how much water needs to be added, weigh a precise quantity (about 20 g) stock alumina into each of six tightly stoppered tubes. Add water by pipette to give a graded series with 4-6.5% water in 0.5% increments (v/w). Shake the tubes vigorously and allow them to equilibrate at least two hours and preferably overnight. Shake again before use, then make into columns as described below. To each of the six columns apply 0.5 ml of a retinol-retinyl ester mixture in hexane, which can be mixed from standard compounds or obtained by partial hydrolysis of the retinyl acetate in the USP reference capsule. Fractionate the sample by the sequential addition of 10 ml portions of pure hexane and of 2, 10 and 50% ethyl ether in hexane. Collect the fractions in ordinary test tubes and view under a hand-held ultraviolet light ( $\sim 360$  nm maximum emission). View the fluorescence through glass to protect the eyes. On a suitably deactivated column the intense milky-green fluorescence of vitamin A appears exclusively in the 2% (retinyl ester) and 50% (retinol) fractions. The correct proportion of water is then added to stock alumina as needed for daily analyses.

Note that storage of stock alumina and partially deactivated alumina for long periods or under humid conditions will change the deactivation characteristics. Thus, the above procedure will need to be repeated periodically.

### *Preparation of Ethyl Ether Free of Peroxides*

Analytical grade ethyl ether is shaken with ferrous sulfate ( $\text{Fe}_2\text{SO}_4$ ) followed by distillation to insure removal of peroxides and antioxidant stabilizers. The redistilled purified ether should be stored in a glass-stoppered, dark bottle over steel wool previously washed with solvent and in a well-ventilated area (preferably under a fume hood). The distillation should be performed using a heating mantle only. Care must be taken to avoid sparks or flames, to provide ventilation (preferably under a fume hood) and to observe strict safety precautions in its preparation, storage and use. Do not store in a closed refrigerator.

### *Preparation of Alumina Columns*

Columns having a bed diameter of about 5 mm and a height of about 4.5 cm are prepared by pouring approximately 1 g of partially deactivated alumina into columns (Pasteur pipettes or the long stem of a funnel can be used) plugged with a small amount of glass wool and with a rubber cap over the opening. The alumina is made into a slurry in degassed hexane and poured into the column to a height of about 4.5 cm. Hexane can be degassed by subjecting a suitable volume ( $\sim 11$ ) to reduced pressure (a water-run

aspirator is sufficient) until bubbles no longer escape from the solvent. The vacuum is gently released and the hexane used within 1-2 hours. Alternatively, the columns can be partially filled with hexane and the alumina added. This procedure prevents the formation of troublesome air bubbles. When the column has settled, the cap is removed and excess hexane is drained and discarded. The sample to be analyzed, dissolved in hexane, is applied before the upper surface becomes dry. At no time during the procedures should the column be permitted to become dry at the upper surface of the alumina.

In general, pure solvent fractions from alumina columns do not give any blue color when treated with the trifluoroacetic acid in chloroform. If subsequent quantitation is to be done by fluorometry, possible contaminants in solvent eluates from the columns must be checked. If a batch of alumina contains fluorescent or other contaminants which give false positive values, it should be rejected.

### Reproducibility

The following results were obtained on four separate control plasmas, based on within-day and between-day variations in retinol and total vitamin A values determined by fluorometry:

Assay Conditions	Plasma	N	Retinol ( $\mu\text{g}/\text{dl}$ )	C.V. (%)	Total Vitamin A ( $\mu\text{g}/\text{dl}$ )	C.V. (%)
Within-day	A	6	$38.7 \pm 1.1$	2.8	$46.0 \pm 2.4$	5.1
Within-day	B	6	$42.2 \pm 1.9$	4.6	$48.8 \pm 1.5$	3.0
Between-days	C	17	$43.6 \pm 2.5$	5.7	$45.9 \pm 2.8$	6.0
Between-days	D	25	$46.5 \pm 3.6$	7.8	$48.6 \pm 3.6$	7.4

## Group Separation by Thin-Layer Chromatography (TLC)

### References

1. Kahan, J. (1967) Thin layer chromatography of vitamin A metabolites in human serum and liver tissue. *J. Chromat.* 30:506.
2. Heaf, D. J., Phythian, B., El-Sayed, M., and Glover, J. (1980) Uptake of retinol, retinol-binding protein and thyroxine-binding prealbumin by egg yolk of Japanese quail. *Int. J. Biochem.* 12:439.

### Principle

This is a microprocedure which makes use of the good resolution possible in separating retinol from its esters by chromatography of the lipid extract on Silica Gel 60 G. The separation is carried out quickly in the presence of antioxidants and involves minimal handling of the sample. Advantage is also taken of the high sensitivity of reflectance spectrophotometry in measuring the amount of the vitamin present in each fraction.

### Apparatus

Glass plates (20 cm  $\times$  20 cm)

Apparatus for spreading adsorbents on thin-layer plates

Glass chromatography tanks for thin-layer plates

Spray gun

Recording spectrophotometer with reflectance  
Scanning accessory  
Microsyringe pipette

## Reagents

Silica Gel 60 G  
Hexane  
Diethyl ether (peroxide free)  
Butylated hydroxy toluene  
5% Solution of arachis oil in light petroleum (40-60°C)  
Known standard solutions of pure retinol and retinyl palmitate

## Procedure

### *Preparation of Thin-Layer Plates*

The glass plates are thoroughly cleaned with chromic acid and distilled water. After drying, six plates at a time are placed on the apparatus for coating with adsorbent. Silica Gel 60 G (26 g) is mixed in a slurry with 50 ml water and placed in the spreading trough, which is moved manually across the plates coating them with a layer of silica gel 0.25 mm thick. After preliminary drying, the plates are transferred to a clean oven at 105°C for one hour. After this reactivation, they are cooled and stored in a closed box containing a desiccant until required for use.

### *Spotting*

Known aliquots (<50  $\mu$ l) of standard samples are applied in spots from a microsyringe in a horizontal line positioned 2 cm from the bottom edge of the plate and spaced 2 cm apart. The spots containing standards of retinol or retinyl palmitate are suitably interspersed between sample spots. The area of the sample deposited at the spot must not exceed 1 cm in diameter. The chromatography tank is lined with Whatman No. 3 paper that dips into the 1 cm layer of chromatography solvent in the bottom of the tank. If Whatman No. 3 paper cannot be obtained, several layers of a thinner paper will serve the same function. This helps to keep the atmosphere of the tank fully saturated with solvent vapor.

### *Chromatography*

The chromatography solvent is prepared by mixing 85 ml hexane with 15 ml diethyl ether and dissolving in it 0.5 g of the antioxidant butylated hydroxy toluene. Solvent to a depth of 1 cm is placed in a chromatography tank. The tank is placed in a dark room or cupboard free from drafts and the tank atmosphere is allowed to become fully saturated with vapor. The lid is slid gently to one side and the plate with samples at the lower edge is placed in the tank and the lid replaced. The solvent is allowed to migrate up the plate for approximately 30 minutes. The plate is removed and a sharp point used to mark the position of the solvent front at both edges and the center. The plate is then lightly and evenly sprayed with a 5% solution of arachis oil in light petroleum as an additional protective antioxidant for retinol during the assay.

### *Scanning*

The solvent evaporates quickly and the plate is mounted on the mobile table of the scanning accessory attached to the spectrophotometer. The photometer is adjusted using a clear piece of the silica Gel so that the recording pen is set at 95% transmittance using ultraviolet light at 325 nm and a slit 0.2 mm wide by 12 mm long. The plate is then

realigned so that each chromatogram of spots can be scanned in turn by the instrument. The presence of retinol or its esters shows as peaks in the recording trace. The areas of these peaks after transformation to absorbance values are directly related to the quantities of retinol present. The standard solutions enable calibration curves to be prepared from which the amounts in the unknown samples can be determined by interpolation. The  $R_f$  values for retinol and the ester fraction are 0.15 and 0.7, respectively. The values for the experimental samples can be checked by viewing the fluorescent spots on the plate under UV light ( $\sim 350$  nm). Alternatively, the plate can be sprayed with a saturated solution of trichloroacetic acid with 0.1% acetic anhydride in chloroform. The pale blue-green color is indicative of retinol.

## Calculation

The height of the recording peak arising from the reduced reflectance due to the absorption of retinol is given by the difference in reflectance between the value at the peak and that at the baseline expressed in absorbance terms using the relationship:

$$\text{absorbance (A)} = \log \left( \frac{100}{\text{Reflectance}} \right)$$

Thus, the peak height is given by  $A_{\text{peak}} - A_{\text{baseline}}$ . The width (W) of the peak at the baseline is determined by measuring the distance between its intersections with the projected slopes of the peak. The approximate peak area is given by:

$$(A_p - A_b) \frac{W}{2}$$

On the basis of areas calculated for known amounts of retinol in standards, a factor (F) is determined which gives ng retinol (or retinyl palmitate)/unit area of peak. Thus the amount of retinol or its ester in unknown samples equals  $F \times \text{Area}$ .

## Comments

The lability of vitamin A to oxidation is well known. When precautions are taken to carry out the chromatography in the dark and with various antioxidants present, the vitamin in practice remains very stable, leading to good recoveries of the material and good reproducibility in the assay. The lower limit of sensitivity of the assay is 10 ng in the aliquot applied to the thin-layer plate. The coefficient of variation among replicates is  $<5\%$ . It is a much more reliable assay than estimating the vitamin by color test, but does require specialized equipment that is relatively expensive.

## Separation of Individual Retinyl Esters by High Pressure Liquid Chromatography and UV Absorbance

### References

1. DeRuyter, M. G. M. and DeLeenheer, A. P. (1978) Simultaneous determination of retinol and retinyl esters in serum or plasma by reversed-phase high performance liquid chromatography. *Clin. Chem.* 24:1920.

2. DeRuyter, M. G. M. and DeLeenheer, A. P. (1979) Effect of silver ions on the reversed phase high performance liquid chromatographic separation of retinyl esters. *Analyt. Chem.* 51:43.
3. Ross, A. C. (1931) Separation of long chain fatty acid esters of retinol by high performance liquid chromatography. *Analyt. Biochem.* 115:324.
4. Bhat, P. V. and Lacroix, A. (1981) Separation and estimation of retinyl fatty acid esters in tissues of the normal rat by high pressure liquid chromatography. *Fed. Proc.* 40:1804. (*J. Lipid Res.*, in press)
5. Huang, H. S. and Goodman, D. S. (1965) Vitamin A carotenoids. I. Intestinal absorption and metabolism of C<sup>14</sup>-labeled vitamin A, alcohol, and  $\beta$ -carotene in the rat. *J. Biol. Chem.* 240:2839.

## Principle

Vitamin A is extracted from plasma (200  $\mu$ l) with methanol:chloroform (2:1), or from tissues with an appropriate organic solvent. After the addition of water, the lower chloroform phase is removed, evaporated under reduced pressure, dissolved in methanol:chloroform (4:1) and sonicated. An aliquot (50  $\mu$ l) is injected into a 10  $\mu$ m octadecyl silica HPLC column followed by methanol as the eluting solvent. Retinol, as detected by its absorption at 330 nm, is eluted first, followed by the following retinyl esters: laurate, myristate, linoleate, palmitate and oleate (together) and stearate. Retinol and its esters are quantitated by use of peak height (or peak area) ratios relative to an internal standard. The retinyl ester concentration in the plasma is normally very low and not quantifiable by HPLC unless first concentrated from larger volumes. The method is best applied to liver, in which retinyl esters, and particularly retinyl palmitate, predominate, but may be used with other tissues as well. It may also be applied to sera collected four hours or more following a massive dose of vitamin A or in conditions where liver disease or hypervitaminosis (both associated with elevated retinyl esters) is suspected.

## Apparatus

Analytical microbalance  
 Centrifuge, desk top  
 Ultraviolet-visible spectrophotometer  
 Roto-evaporator  
 Vortex mixer  
 Ultrasonic bath  
 High performance liquid chromatography system, equipped with a variable wavelength detector  
 Octadecylsilane chromatographic column (18% bonded organic phase) or other appropriate columns  
 Nitrogen or argon  
 Water bath, 40-50° C  
 Micropipettes (10  $\mu$ l, 15  $\mu$ l, 50  $\mu$ l, 100  $\mu$ l)  
 Glass test tubes (10  $\times$  75 mm) or conical centrifuge tubes (14  $\times$  100 mm) with Teflon-lined screw caps  
 Pasteur pipettes  
 Hamilton 710 syringe or a similar product

## Reagents

Retinyl propionate, all-*trans*  
Retinyl acetate, all-*trans*  
Retinyl palmitate, all-*trans*  
Methanol, analytical grade  
Chloroform, analytical grade  
Pyridine, reagent grade

## Procedure (DeRuyter and DeLeenheer, 1978)

### *Extraction*

Transfer 200  $\mu\text{l}$  of serum (or plasma) to a centrifuge tube; add 0.6 ml of water, 2.0 ml of methanolic solution of internal standard (retinyl propionate: 137  $\mu\text{g}/\text{l}$ ), and 1.0 ml of  $\text{CHCl}_3$ . After thorough mixing for one minute and standing for five minutes, add 1.0 ml of water and 1.0 ml of  $\text{CHCl}_3$ . Mix gently and centrifuge ( $1500 \times g$ , 5 minutes). Transfer the  $\text{CHCl}_3$  (lower) phase to an evaporation tube and evaporate under reduced pressure. Dissolve the residue in 100  $\mu\text{l}$  of  $\text{CH}_3\text{OH}/\text{CHCl}_3$  (4/1 by vol). After sonication until the residue is solubilized ( $\leq 10$  minutes), inject 50  $\mu\text{l}$  of the solution on top of the column. Use methanol as the eluting solvent.

### *Chromatographic Conditions*

Column	Octadecylsilane chromatographic column with 18% bonded organic material
Mobile phase	Absolute methanol
Flow rate	1 ml/minute
Pressure	Not specified
Detector wavelength	330 nm
Detection sensitivity	0.1 AUFS* (retinol), 0.01 AUFS (retinyl esters)
Temperature	Ambient
Elution times for:	Minutes:
Retinol	1.6
Retinyl propionate	2.1
Retinyl laurate	5.8
Retinyl myristate	7.9
Retinyl linoleate	8.7
Retinyl palmitate	11.0
Retinyl oleate	11.0
Retinyl stearate	15.4

\*AUFS: Absorption units full scale.

### *Analysis by the Peak Height (or Area) Ratio Procedure*

The use of an internal standard, such as retinyl propionate or retinyl laurate, allows automatic correction of losses and inaccuracies in pipetting. The principle of the procedure is explained in detail on page 16. Calibration curves are run for different concentrations of retinol, retinyl palmitate, retinyl linoleate, retinyl stearate and any other esters of interest together with a fixed amount of the internal standard. Peak height ratios are plotted as the ordinate (height of the vitamin A compound of interest divided by the internal standard) versus mass (or molar) ratios as the abscissa. From the mass ratio found, the amount of internal standard added and the aliquot used, the concentration of the vitamin A compound of interest in the plasma can be calculated.

### *Standards*

The  $E_{325}^{1\%}$  values at 325 nm in hexane for all-*trans* retinol and retinyl acetate are 1850 and 1610, respectively. Calculated  $E_{325}^{1\%}$  values of other esters in hexane are as follows: retinyl propionate (1547), retinyl linoleate (966), retinyl palmitate (1010), retinyl oleate (962), and retinyl stearate (959).

Acyl esters of retinol can be synthesized as follows: 10  $\mu$ g of all-*trans* retinol and 500  $\mu$ l of pyridine are mixed in a small test tube in dim light followed by 15  $\mu$ l of the appropriate fatty acyl chloride. The test tube is then kept at 50-55°C in the dark under nitrogen for one hour. Pyridine is carefully and completely evaporated off under nitrogen at 40-50°C and the residue is dissolved in hexane. Alternatively, the retinyl ester can be dissolved in 1 ml hexane and the pyridine washed out with several 1 ml rinses of 0.1 N HCl, of 0.1 N NaOH in 50% ethanol and then of water. The hexane solution is then applied directly to a 6% (v/w) water-deactivated alumina column (1.5 cm diameter  $\times$  10 cm). After washing further with hexane (20 ml), the retinyl ester is eluted with 2% acetone in hexane. A suitable amount of ethyl ether in hexane may also be used as the eluant. Retinyl esters might be further purified on HPLC. The yield of most esters is 75-95%.

### *Reproducibility*

	<u>Retinol*</u>	<u>Retinol*</u>	<u>Retinyl palmitate<sup>†</sup></u>	<u>Retinyl stearate<sup>†</sup></u>
Type	Between-days	Within-day	Within-day	Within-day
$\bar{x}$	65 $\mu$ g/dl	72 $\mu$ g/dl	646 $\mu$ g/dl	415 $\mu$ g/dl
C.V.	4.9%	2.0%	4.3%	6.0%

\*Different plasma samples were analyzed.

<sup>†</sup>These studies with esters were conducted with serum from a subject who had ingested 300,000 IU of vitamin A four hours earlier.

### *Sensitivity*

The lower detection limit, using 200  $\mu$ l plasma, is 5  $\mu$ g retinol/dl and 10  $\mu$ g of retinol in ester form/dl. In blood from fasting subjects the retinyl ester fraction is normally too low to be detected unless larger plasma samples are drawn or the ester fraction is first concentrated.

### **Effect of Sample Storage**

Serum samples kept for a month in the dark at -18°C gave the same values.

### **Variations and Modifications**

The method has been applied to the analysis of retinyl esters in lymph chylomicra<sup>3</sup> and

in tissues, including liver<sup>1</sup>. In the analysis of tissues, the tissue sample was washed in cold phosphate buffer, blotted and lyophilized overnight. The tissue was ground to a powder in a mortar and pestle, extracted with 20 ml 99% methanol per g tissue and then with 50 ml hexane. The methanol and hexane extracts were separately evaporated and the residues combined and redissolved in a small volume of chloroform:methanol (1:1). An aliquot was then analyzed by HPLC on a 5 $\mu$  ultrasphere octadecylsilane (ODS) column.

The separation of retinyl oleate and retinyl palmitate poses a problem. By incorporating silver ions (0.06 M AgNO<sub>3</sub>) into the mobile phase (methanol), the retention times of unsaturated fatty acyl esters but not of saturated esters are reduced. Thus the retention time for retinyl oleate is reduced from 11 minutes to seven minutes and for retinyl linoleate from nine minutes to five minutes in the presence of 0.06 M AgNO<sub>3</sub>. Separation of the oleate and palmitate esters may also be achieved by use of a homogeneous column of very fine particle size, either a 5 $\mu$  Supercosil LC-8 column<sup>2</sup> or a 5 $\mu$  Ultrasphere ODS column<sup>1</sup>. On such columns retinyl palmitate elutes slightly before retinyl oleate, although the retention times are long, e.g., 120 and 130 minutes<sup>3</sup> and 57 and 59 minutes<sup>1</sup>, respectively.

## Comments and Precautions

In order to separate retinol and individual retinyl esters on the same column in a relatively short period (<20 minutes), heavy loading of the column ( $\geq 18\%$ ) with organic phase is necessary.

Although ethanol followed by hexane has often been used in extracting retinol from plasma, a more complete extraction is obtained by using chloroform-methanol (2:1). After evaporation of the extracting solvent, the lipid residues from serum or liver do not dissolve readily in methanol. The presence of some chloroform in the methanol solution is usually necessary, and the process of solution can be speeded by sonication or by warming. Fortunately, the presence of chloroform does not overly affect the retention behavior of the esters.

As an internal standard for retinyl esters alone, retinyl laurate, retinyl myristate or retinyl heptanoate is suitable. These esters are found, at most, in miniscule quantities in blood and tissues, yet have a retention time closer to that of the fatty acyl esters of longer chain length that are usually present.

In biological materials containing high fat concentrations relative to retinyl esters, a preliminary separation of retinyl (and cholesteryl) esters from triglycerides on an alumina column is advisable.

The separation of retinyl esters by these methods must for the present be considered primarily as a research tool. Since the composition of fatty acids in retinyl ester is controlled metabolically and is not much affected by dietary intake, the retinyl ester composition has few nutritional implications.

## Retinol-Binding Protein (RBP)

### Total RBP by Immunoassay (Radial Immunodiffusion)

#### References

1. Mancini, G., Carbonara, A. O., and Heremans, J. F. (1965) Immunochemical

- quantitation of antigens by single radial immunodiffusion. *Immunochem.* 2:235.
2. Ingenbleek, Y., van den Schriek, H. G., DeNayer, P., and DeVisscher, M. (1975) The role of retinol-binding protein in protein-calorie malnutrition. *Metabolism* 24:633.
  3. Glover, J., Moxley, L., Muhilal, H., and Weston, S. (1974) Micromethod for fluorometric assay of retinol-binding protein in blood plasma. *Clin. Chim. Acta* 50:371.
  4. Venkataswamy, G., Glover, J., Cobby, M., and Pirie, A. (1977) Retinol-binding protein in serum of xerophthalmic, malnourished children before and after treatment at a nutrition center. *Amer. J. Clin. Nutr.* 30:1968.
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  6. Heaf, D. J. and Glover, J. (1979) Circannual changes in plasma concentrations of immunoreactive retinol-binding protein and luteinizing hormone in male and female Japanese quail. *J. Endocr.* 83:323.
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  8. Peterson, P. A. (1971) Characteristics of a vitamin A-transporting protein complex occurring in human serum. *J. Biol. Chem.* 246:34.
  9. Futterman, S. and Heller, J. (1972) The enhancement of fluorescence and the decreased susceptibility to enzymatic oxidation of retinol complexed with bovine serum albumin,  $\beta$ -lactoglobulin and the retinol-binding protein of human plasma. *J. Biol. Chem.* 247:5168.
  10. Kirby, W. Ph.D. thesis, University of Liverpool, 1971.

## Principle

The radial immunodiffusion procedure<sup>1</sup> has been used to assay retinol-binding protein (RBP) in serum or plasma in many laboratories. The RBP as a protein antigen is allowed to diffuse from the sample placed in a small cylindrical well cut into a thin layer of gel containing monospecific antiserum. When the concentration of the spreading antigen falls appreciably below that of the uniformly distributed specific antibodies, the two interact to form a precipitate in the form of a halo or ring around the well which marks the end point of their reaction. The precipitated protein (or precipitin) can be visualized in the gel by illuminating it from the side and viewing the scattered light against a dark background, or by staining the precipitate with a suitable protein dye after washing the residual soluble proteins away.

The area of the ring of precipitin is directly related to the concentration of diffusing antigen from the sample. By running solutions of known concentration alongside samples on each layer of gel, it is possible to prepare a calibration curve from which the unknowns can be determined. It is customary to measure the diameter of the rings in two directions at right angles and then to plot their product directly against the concentration of the RBP in the different standards.

## Apparatus

Gel plates: available commercially\* in a plastic tray with snap fitting lid containing 12

\*Immunodiffusion kits for determining retinol-binding protein by radial immunodiffusion are obtainable from the Calbiochem-Behring Corporation, 10933 North Torrey Pines Road, La Jolla, California 92037. The kits come complete with standardized human serum for use as a reference standard. The plates are relatively stable but should be stored cool in a refrigerator between 2° and 8° C.

wells in an annular ring of gel. These kits are very convenient when small groups of samples are being measured. However, in a large survey, particularly in regions where the concentration of retinol-binding protein is suspected to be very low, it is frequently more economical to prepare thin layers of gel freshly on larger rectangular glass plates (either 10 or 20 cm × 8 cm) that are capable of handling about 50 or 100 samples at a time. The wells are made smaller (3 mm diameter) and the volume of sample required for assay need only be 3 to 5  $\mu$ l.

Microsyringe pipette: 0-50  $\mu$ l capacity

Sealable container to hold the trays during the diffusion period, supplied in the commercial kit.

Shallow glass (crystallizing) dishes for washing surplus protein out of the gel and for staining the precipitin.

Calibrated magnifier to measure the precipitin rings to 0.05 mm.

Illuminator to show up the rings for measurement. (Fluorescent tube covered with an opal glass plate suffices.)

## Reagents

Phosphate buffered saline (0.05M potassium phosphate, pH 7.4 containing 0.15 M NaCl)

Staining solution:

Coomassie Blue (Kenacid Blue)	0.5 g	Dissolve the dye in the solvent mixture and filter before use
Glacial acetic acid	20 ml	
Methanol	100 ml	
Water	80 ml	

Destaining solution:

Glacial acetic acid	28 ml	Used destaining solution can be regenerated by passing it through a filter bed of charcoal
Methanol	80 ml	
Water	320 ml	

Agarose (purified and electrophoresis)

Stock barbital buffer at pH 8.6:

Sodium diethyl barbital	20.6 g
Barbituric acid	4.0 g
Sodium azide	1.6 g
Water	1000 ml

*Standard solution of holo-RBP:* Pure holo-RBP, which consists of a 1:1 molecular complex of all-*trans* retinol and RBP, has been isolated from human plasma by standard protein fractionation procedures.<sup>7,8</sup> Traces of apo-RBP can be removed by electrophoresis on polyacrylamide disc gels. The  $E_{278}^{1\%}$  value of pure holo-RBP at 278 nm in isotonic saline at pH 7.4 is 17.5,<sup>9</sup> and at 328 nm is the same. The concentration of pure holo-RBP in a plasma sample can be estimated using this absorbance value.

*Preparation of a Reference Standard Using Human Plasma:* Standard holo-RBP is used in determining the concentration of total retinol-binding protein in a large reference sample (50-100 ml) of human plasma by radial immunodiffusion against the specific antiserum to holo-RBP raised in the rabbit.<sup>3</sup> In comparing the concentration of RBP in the reference plasma sample with that in the standard solution, 3% pure bovine serum albumin should be added to the standard to raise the total protein content. This is necessary in order to make the conditions of migration of the two samples comparable. Human serum albumin is *not* used since some preparations of it contain traces of RBP as an impurity. After standardization, the human reference plasma is stabilized with 0.1%

sodium azide, divided into 0.2 ml portions in tightly sealed vials, and stored in the frozen state at  $-20^{\circ}\text{C}$ . The use of small independent portions that can be thawed as needed avoids the deleterious effects of repeated thawing and freezing.

## Procedure

### *Preparation of Agarose Gel Plates*

The stock buffer is prepared by dissolving the barbituric acid in 600 ml distilled water by heating it to near boiling point. The sodium diethyl barbitol and azide are then added in turn, the mixture allowed to cool and made up to 1 liter. This solution is diluted five times before use in preparing the gel plates.

The agarose gel layer on the glass plate is prepared as follows:

1. The agarose (1.5 g) is gradually added to 100 ml warm buffer solution and the mixture brought to a boil in a small beaker on a hotplate. This is sufficient to cover four glass plates  $20\text{ cm} \times 8\text{ cm}$ . Smaller quantities are taken for smaller plates.

2. To the appropriate volume of agarose solution, after it has *cooled to  $50^{\circ}\text{C}$* , is added the correct amount of monospecific antiserum to RBP (usually  $50\text{-}100\ \mu\text{l}$  depending on its titer) with gentle stirring to avoid trapping air bubbles.

3. The clean glass plates are previously mounted on a perfectly *level* table and preheated with an overhead lamp to about  $40^{\circ}\text{C}$ . The agarose solution is then poured carefully over the whole of the plate and allowed to cool. The gel sets in 10-15 minutes and forms a uniform layer just less than 2 mm thick.

4. Wells (3mm diameter) are punched out of the gel 1 cm apart along rows spaced 1 cm apart. These wells will take samples up to a  $5\ \mu\text{l}$  volume.

### *Sample Treatment*

The  $5\ \mu\text{l}$  samples of the serum or plasma to be assayed are applied to the wells in duplicate using a micropipette, while the plate is mounted above a grid showing the sample order numbers. A number of the wells from each row are chosen randomly to accommodate the standard human serum and three appropriate dilutions of the standard serum are used to cover the expected range of RBP. The unknowns are pipetted into the remaining wells and the gel marked by cutting off corners to help identify their placement in the series. The tray or plate with samples is then placed horizontally into a sealable plastic box containing some moistened filter paper to maintain a saturated atmosphere. The sealed box is placed in a warm incubator or room at  $37^{\circ}\text{C}$  for approximately 36 hours so that the antigen can diffuse into the gel and interact with the antiserum. The plate is removed and soaked in a glass dish with physiological saline overnight to wash out the residual soluble protein. A second wash is carried out for 15 minutes with distilled water containing a little glycerol. The gels are dried by pressing them with filter paper and warming in an oven or with a hair dryer. The dried gel still attached to their plates is stained in Coomassie blue stain for 10-15 minutes and then destained in the destaining solution for several hours when the precipitin halos around the wells will be clearly seen against a pale blue background.

The diameters of the halos can be read with a magnifier fitted with a graticule (grid, divided into squares) after the stained plate has dried and been placed on top of a diffuse light source.

## Calculations

A calibration curve is prepared using at least four dilutions of the standard serum in order to bridge the concentration range within which it is expected the unknown will fall.

The unknown values are read off from the best fit curve through the standard points. The assays are carried out in duplicate and the coefficient of variation is usually <3%.

## Comment

Plasma of well-nourished persons contains a small proportion of apo-RBP in addition to holo-RBP. Since both types of protein interact similarly with antibodies, the value found by this method gives the total immunoreactive protein in the sample. In many vitamin A-deficient children, the holo-RBP can decline to zero whereas apo-RBP may remain constant or even increase. Hence, the total RBP value does *not* give an assessment of the degree of saturation of the protein with retinol. Furthermore, as deficiency develops, the rate of secretion of the native protein from the liver is inhibited, further complicating the interpretation of total RBP values.

Total plasma RBP measurements are consequently most valuable in the vitamin A-replete subject, in which it gives a reliable measure of the physiologically active retinol in the blood. It has also proved useful in monitoring the recovery of malnourished children following treatment with retinol and an improved protein diet.

In normal adults the range of total RBP concentrations can vary from 4-9.0 mg/dl. In normal preschool children, values are generally within the range from 2.5-3.5 mg/dl. In vitamin A-deficient children, however, the concentration often falls to about 1.0 mg/dl. In such severe cases retinol (holo-RBP) is virtually absent from the plasma while some apo-RBP may remain.

It should be noted that total RBP values are reduced by prolonged restriction of calories, as well as of protein. These variables must be considered in the interpretation of RBP values as a measure of vitamin A nutriture.

## Total RBP by Electroimmunoassay

### References

1. Laurell, C.-B. (1966) Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Analyt. Biochem.* 15:45.
2. Glover, J., Neal, G., and Carrol, J. unpublished method.

### Principle

In this method the migration of the antigen is directed from the well into the thin layer of gel containing the antiserum by an electric current. The immunoglobulins scarcely move so that the precipitin forms well beyond the well in the form of a cone or "rocket", the height and width of which can be easily measured. The area of the cone is directly related to the concentration of antigen present.

This procedure, which is more sensitive than that of radial immunodiffusion, is recommended where the concentration of RBP is very low and the halo of precipitin in radial immunodiffusion does not extend appreciably beyond the edge of the well, thereby making the measurement of its diameter difficult. Electroimmunoassay is also more rapid for a small number of samples.

## Apparatus

Rectangular glass plates (10 or 20 cm × 8 cm) as used for radial immunodiffusion (RID)  
Hollow punch (type) or fine glass capillary, of the same diameter connected to a water vacuum pump  
A level surface: a firm plastic board fitted with three leveling screws suffices  
Spirit level  
Portable lamp  
Hot water bath  
Flat bed electrophoresis tank fitted with water cooling jacket  
Stabilized electric power supply (100 ma at 500V)  
Hairdryer  
Magnifier with calibrated graticule to 0.05 mm  
Fluorescent illuminator

## Reagents

Phosphate buffered saline  
Staining solution\*  
Destaining solution\*  
Agarose (purified for electrophoresis)  
Stock barbital buffer at pH 5.6\*  
Standard solution of holo-RBP\*  
Standardization of human plasma\*

\*Same as used in radial immunodiffusion (p. 46)

## Procedure

### *Preparation of Agarose Gel Plates*

The gel plates are prepared in the same way as for RID above, but the wells are cut only 0.8 cm apart in a single row 1.5 cm from one of the longer edges.

### *Electroimmunoassay*

The prepared plate is placed on the water cooled block of the electrophoresis tank and the connecting paper wicks (or other absorbent material) laid along the longer edge of each side of the plate. A glass rod is placed on top of each to ensure good contact between the wick and the gel. The samples and four different dilutions of standards (5  $\mu$ l each) are inserted into the wells lying next to the cathode and the lid of the tank is replaced. The proteins are subjected to a potential of 100V and a current of 1.5 ma/cm for five hours to drive them into the agarose towards the anode. A cone or rocket shaped precipitin band is formed, which can be revealed by protein staining. The plates are removed and washed with saline before immersion in the stain as previously described. After destaining, the heights and base widths of the rockets are measured using the magnifier. Both height and base width measurements are made with the upper edge of the well as reference point.

## Calculation

The product of height × base width approximates twice the area of a rocket. These double areas of the rockets for the standard solutions are plotted against their known concentrations, and the RBP concentrations of the unknowns are obtained by

interpolation from the best fit line through the points. Assays are carried out in duplicate. The coefficient of variation is <8%.

## Comment

In preparing the gel plates great care must be exercised not to overheat the antiserum. The antiserum concentration can be adjusted slightly to enable an appropriately sized cone of precipitin to be formed for the concentration range of antigen to be encountered. The size of the well can be reduced to 2 mm diameter, so that more samples can be accommodated on one plate. It is not advisable to make the well too small, however, in view of the increasing error which occurs in pipetting very small samples. If too strong an electric current is used, the migrating antigen will not interact satisfactorily with its specific antibodies. Since the electrophoretic mobility of RBP is 0.6 that of albumin, the protein should have migrated sufficiently across the plate in 4-5 hours to achieve optimal precipitation. The quantity of antiserum dispersed in the gel must be sufficient to ensure complete precipitation of the encountered antigen, when the latter has migrated about 3 cm from the well during the five-hour period. If not, the standard curve will not be linear at the higher concentration range. The method is particularly suitable for plasma samples which contain <2 mg RBP dl.

## Holo-RBP by Fluorometry

### References

1. Glover, J., Moxley, L., Muhilal, H., and Weston, S. (1974) Micromethod for fluorometric assay of retinol-binding protein in blood plasma. *Clin. Chim. Acta* 50:371.
2. Glover, J. (1980) Fluorescence assay of retinol-binding holo-protein methods. In: *Methods in Enzymology*. Vol. 67, D. B. McCormick and L. D. Wright, eds., New York: Academic Press.
3. Davis, B. J. (1964) Disk electrophoresis. II. Methods and application to human serum proteins. *Ann. N. Y. Acad. Sci.* 121:404.

### Principle

Holo-RBP is first separated from other plasma proteins as much as possible by electrophoresis on polyacrylamide gel and then is quantitatively analyzed by fluorometry. Advantage is taken of the following facts: (1) that holo-RBP has an electrophoretic mobility in the  $\alpha$ -region, which is well separated from other major proteins, (2) that the fluorescence of retinol is 14 times stronger when bound to RBP than when dissolved in a hydrocarbon solvent, and (3) that retinol is very stable while attached to its carrier. Plasma retinyl esters, which are associated with the low density lipoproteins, are well separated from holo-RBP. Thus by scanning the region of the gel containing holo-RBP with ultraviolet light, it is possible quantitatively to assay the fluorescence of this physiologically active form of the vitamin.

### Apparatus

Standard disc-gel electrophoresis tank and power supply

Standard disc-gel tubes: 7 cm long  $\times$  0.6 cm internal diameter. Gel tubes must be cleaned with chromic acid and distilled water before use; otherwise acrylamide gel will not adhere properly to glass.

Chromascan recording fluorophotometer with UV light source  
 Rectangular quartz cell (12 × 0.8 × 1.8 cm deep) in which the gel is placed for scanning

## Reagents

*Stock Solutions:*

20% acrylamide	
Acrylamide monomer	200 g
Bisacrylamide	6.7 g
Water	1 liter

Filter solution before use.

### *Resolving gel buffer:*

1 M Hydrochloric acid	240 ml (20 ml concentrated HCl)
Tris*	181.5 g
TEMED†	3 ml
Distilled water	1 liter

\*NNN'N' - Tetramethylene diamine

†NNN'N' - Tetramethyl-1,2-diaminoethane

### *Stock buffer solutions for eletrophoresis:*

<i>1 M Tris-HCl Buffer pH 8.1</i>		<i>1 M Tris-Glycine Buffer pH 8.9</i>	
Tris	121.14 g	Tris	121.14 g
Conc. HCl	46 ml	Glycine	78.8 g
Distilled water	1 liter	Distilled water	1 liter

Dilute the above stock buffer 10 times to prepare 0.1 M Tris-HCl to use in the *lower* compartment

Dilute the above stock buffer 20 times to prepare 0.05 M Tris-glycine to use in the *upper* compartment

### *Preparation of gels:*

	<i>Resolving gel (5%)</i>	<i>Concentrating gel (4%)</i>
20% acrylamide	16 ml	2 ml
Resolving gel buffer	22 ml	1 ml
Ammonium persulphate in 10% sucrose	22 ml of a freshly prepared 0.4% solution	3 ml of a freshly prepared 0.6% solution
Distilled water	0	4 ml
Sufficient solution for:	8 gels - 6.0 cm long	22 gels - 1 cm long

*Standardized human serum* (as used in radioimmunodiffusion)

*Preparation of samples:* To increase the viscosity and density of the samples for layering onto the top of the gel, an equal volume of 20% sucrose is added to a portion prior to analysis.

## Procedure

### *Gel Preparation*

Polyacrylamide disc gels are prepared in a manner similar to that described by Davis.<sup>3</sup> The gel tubes mounted vertically in their rubber supports are filled to within 2 cm of the top using 5% (w/v) polyacrylamide resolving gel containing 0.75 M Tris-HCl buffer at pH 8.9 and 3.5% (w/v) sucrose. The gel is then carefully covered with water until the gel has set (10-15 minutes). The layer of water is removed and a further 1 cm of 4% (w/v) polyacrylamide concentrating gel containing 0.25 M Tris-HCl of the same pH is added. This in turn is covered with water until it sets. After 15-20 minutes the water can be decanted off and the requisite number of gel tubes inserted to a uniform height into the electrophoresis tank, the lower buffer compartment of which is filled with 0.1 M Tris-HCl at pH 8.1. Care is taken to ensure that air bubbles are not trapped at the base of the glass tubes. The Tris-glycine buffer at pH 8.9 is then poured into the upper compartment so that the tops of the tubes are covered to a depth of about 1 cm. Again, air bubbles must not be left in the tubes. The electrophoresis tank is then transferred to a cold cabinet or cold room at 4°C.

### *Electrophoresis*

When the gels have cooled, the samples of unknown and one standard containing 10% (w/v) sucrose are applied gently as a thin layer to the gel-upper buffer interface using a microsyringe pipette. Sample volumes of 5-20  $\mu$ l are used depending on the expected concentration of holo-RBP. The standard plasma serves as a control check on the performance of the instrument during the assay. A constant current of 4 ma/tube is applied for 90 minutes across the tubes by which time the holo-RBP should have penetrated about 3 cm into the resolving gel as a sharp zone about 3 mm wide. The position of the holo-RBP can be seen by irradiating the tubes briefly with ultraviolet light (350 nm) in the darkroom.

### *Estimation of Fluorescence*

When the holo-RBP has traveled about 3 cm into the gel, the tubes are removed from the tank. Each gel is expelled in turn from its tube by inserting a fine syringe needle a short distance down the side and applying some water pressure around the gel to ease it out for placement in the quartz cuvette. The gel is covered with distilled water to a depth of 3-4 mm and the cuvette mounted in its holder for insertion into the chromascan. The end of the resolving gel is placed in the light path and the baseline control of the instrument adjusted so that the recording pen is approximately 1 cm above the stop position. The zone of the gel containing the holo-RBP is scanned using ultraviolet light (Hg arc with Woods glass filter) and a slit 0.5 mm wide by 0.7 cm long, which is just sufficient for the beam to irradiate the full diameter of the gel. A second scan is run after turning the gel through 180° in the cuvette, just in case the protein may have run unevenly through the gel. The areas of the two peaks on the recording are measured by triangulation and the mean area taken as the value for the sample. A series of dilutions of standard sera or plasma containing known amounts of retinol are also run to prepare a standard calibration curve from which a correction factor relating retinol concentration to that of holo-RBP can be derived.

The amount of holo-RBP applied to the gel normally should not exceed 1  $\mu$ g, i.e., 20  $\mu$ l of a 50  $\mu$ g/ml preparation; otherwise there is loss of linearity between fluorescence and concentration of retinol due to self-absorption effects.

## Calculation

The double peak areas (height  $\times$  base width) for the standard solutions are plotted against the concentration of retinol expressed in nmoles/ml and the line of best fit determined. The slope gives double the fluorescence peak area per nmole retinol/ml, i.e., double the fluorescent peak area per nmole holo-RBP/ml, since 1 mole of retinol binds to 1 mole of RBP. On the basis of a molecular weight for RBP of 21,500 daltons, the double fluorescence peak area is:

$$A \text{ cm}^2 = 1 \text{ nmole RBP/ml} \equiv 21.5 \text{ } \mu\text{g RBP/ml or } 2.15 \text{ mg/dl}$$

then:

$$A/21.5 \text{ cm}^2 \equiv 1 \text{ } \mu\text{g RBP/ml or } 0.1 \text{ mg/dl}$$

This is the conversion factor for transforming double peak areas of unknown samples into concentrations of holo-RBP.

## Comments

The method is rapid, reproducible, highly sensitive and measures solely holo-RBP. The stability of retinol under the assay conditions is excellent. When care is taken in applying the sample to the gel, the coefficients of variation for within-run replicates is  $<3\%$  and for assays done over a long period is  $7\%$ . Reproducibility is affected, however, by variations in the energy of the light source or minor changes in background fluorescence. Since sensitivity is affected by the type of buffer used in making the gel, the buffer used for a set of analysis should be the same.

Partially hemolyzed samples are difficult to analyze, inasmuch as hemoglobin migrates close to holo-RBP and distorts the base line obtained in the fluorescence scan. The major constraint in utilizing this method is the limited availability of the scanning fluorometer. In addition, this instrument is reasonably expensive and requires a stable electrical source and careful maintenance to yield satisfactory quantitative results.

## Holo- and Apo-RBP by Electroimmunoassay

### Reference and Introduction

1. Glover, J., Neal, D., and Carroll, J. Unpublished method.

This procedure was developed to enable both holo- and apo-forms of RBP to be determined directly by use of simple hospital laboratory equipment. A good reference serum must be available, however, with known concentrations of the two forms of RBP. Hitherto the concentration of the apoprotein has been obtained by taking the difference between the concentration of total RBP by immunoassay and that of the holoprotein by fluorometric assay. The latter procedure, described on page 50, requires the use of an expensive spectrophotometer and scanning fluorimeter, which are not widely available.

### Principle

Holo- and apo-RBP are resolved by subjecting the plasma sample to electrophoresis on thinner (3-4 mm diameter) than normal ( $\sim 8$  mm) polyacrylamide disc-gel cylinders. The

apoprotein has a greater net negative charge than the holoprotein and migrates just in advance of the latter. The gel is extruded from its supporting tube and the position of the holoprotein is noted by its fluorescence when exposed to ultraviolet light at 354 nm. This section of the gel (3 mm wide) is cut out with a sharp scalpel or razor blade, together with 3 mm sections immediately behind and in advance of the fluorescent zone. These sections are then placed in order in a single row of 4 mm diameter wells of an anti-RBP containing agarose plate and subjected to electrophoresis. The areas of the cone shaped precipitin bands are proportional to the amounts of holo-RBP or apo-RBP present.

## Apparatus

Disc-gel electrophoresis tank fitted with narrow bore grommets to take thin gel tubes (4 mm diameter  $\times$  7 cm long)

Flat bed electrophoresis tank fitted with a water cooled support block for the gel plates

Apparatus for making thin-layer gel plates (pps. 38-39)

Glassware for staining and destaining plates (pps. 45-46)

Magnifier and illuminator for measuring areas of precipitins

## Reagents

Reagents are the same as those used for disc-gel preparation (p. 51) and for immunoelectrophoresis (pps. 49 and 51).

## Procedure

Preparation of polyacrylamide disc-gels and the agarose gels for immunoassay is carried out as previously described on pages 47 and 52. The thin vertical polyacrylamide disc-gels are prepared in the 4 mm diameter tubes and mounted to a uniform depth in the apertures of the upper compartment of the electrophoresis apparatus. If narrower bore grommets are not available to replace the standard ones for the wider 8 mm diameter tubes, then a piece of adhesive tape wrapped around the thinner tube will increase the diameter sufficiently to make a good seal.

The apparatus is assembled, filled with the appropriate buffer solutions and transferred to the cold room or cabinet as indicated on page 52. Samples (5  $\mu$ l) of plasma to which 10% sucrose has been added are gently layered on top of the buffer/gel interface using a microsyringe pipette. Then a current of 2 ma/tube is applied for 100 minutes across the series of tubes, by which time holo- and apo-RBP will have penetrated just over 3 cm into the gel. The position of the holo-RBP can be detected using UV light at 354 nm in the dark. The gels are extruded from the tubes using gentle water pressure from a syringe and placed in a glass tray.

With the UV light as a marker, the fluorescent 3 mm section (No. 2) of the gel containing holo-RBP is cut out with a sharp blade, together with the 3 mm section (No. 1) immediately behind and two 3 mm sections (Nos. 3 and 4) immediately in advance of the fluorescent zone. The four pieces are then transferred in prescribed order to the 4 mm wells of a 2 mm thick agarose gel immunoelectrophoresis plate (20 cm  $\times$  8 cm), which has been placed in the flat electrophoresis tank. Four dilutions of a known working standard serum are also placed in some of the other wells. Wicks are applied and weighted down with glass rods. A current of 1.5 ma/cm is applied for five hours to drive the proteins out of the polyacrylamide and into the agarose gel. As previously noted on page 49, the antigens precipitate in the form of cones or rockets.

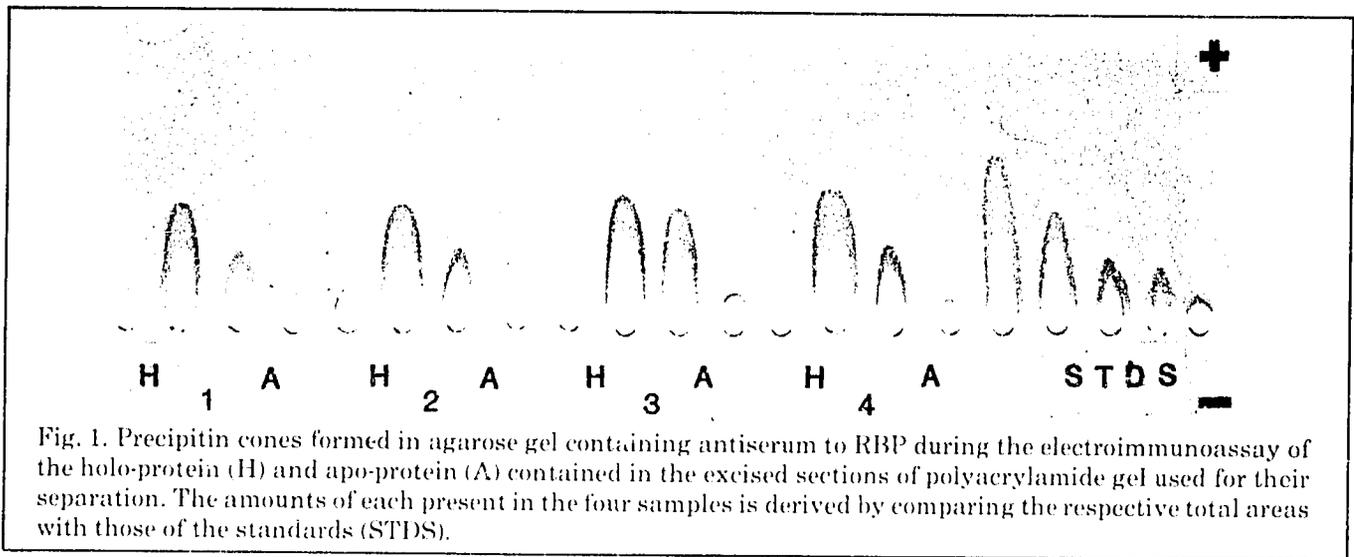


Fig. 1. Precipitin cones formed in agarose gel containing antiserum to RBP during the electroimmunoassay of the holo-protein (H) and apo-protein (A) contained in the excised sections of polyacrylamide gel used for their separation. The amounts of each present in the four samples is derived by comparing the respective total areas with those of the standards (STDS).

The plate is removed and soaked in physiological saline containing 0.02% azide for at least 16 hours to extract the residual soluble proteins, after which it is rinsed with distilled water and dried in a warm air stream. The gel is then stained as previously described on page 47 and the areas of the precipitin cones measured. A sample plate is shown in Fig. 1. The amount of holo-RBP is given by the combined areas of section Nos. 1 and 2 and the amount of apo-RBP by the areas of section Nos. 3 and 4, respectively.

## Calculation

A calibration curve is plotted of the products of width  $\times$  height for the cones as a function of the RBP concentration in the standards. The results for the unknown samples are obtained by interpolation from the curve. Alternatively, the percentage areas corresponding to holo- and apo-protein may be used to calculate the proportions present in total RBP, which has been assayed directly by immunodiffusion or electroimmunoassay methods.

## Comments

This procedure gives the same value for holo-RBP as that obtained by the fluorescence procedure within an experimental error of 5% (Table 3). Although not as rapid as the former, it does not require the expensive fluorophotometer. The within-run coefficient of variation is  $<8\%$ . After practice in running the gels, two sections of 4 mm can be cut instead of four of 3 mm; the first contains essentially all the holoprotein and the second all the apoprotein. This modification obviously enables more samples to be examined on a single agarose plate.

TABLE 3.  
The Concentration of Holo-RBP in Plasma Samples Determined by Electroimmunoassay (EIA) and by Fluorescence (F) Procedures

Human Plasma Sample Nos.	Total RBP by EIA mg/dl ( $\pm$ SEM)	Holo-RBP	
		By EIA mg dl ( $\pm$ SEM)	By F
1	9.0 $\pm$ 0.20 (5)*	5.0 $\pm$ .07 (5)	4.9 (2)
2	8.5 $\pm$ 0.18 (5)	4.6 $\pm$ .10 (5)	4.2 (2)

\*Figures in parentheses indicate number of replicates.

Another technique for estimating the relative amounts of apo- and holo-RBP is to measure the molar ratio of retinol to total RBP in plasma. Since holo-RBP has a 1:1 molar ratio of retinol and RBP, any ratio  $<1.0$  is due to the presence of apo-RBP.

## Total Vitamin A in Human Liver Samples

### Reference

1. Olson, J. A. (1979) A simple dual assay for vitamin A and carotenoids in human liver. *Nutr. Reports Intern.* 19:807.

### Principle

A weighed fresh or frozen sample of liver is mashed in a vial with anhydrous sodium sulfate, covered with a known volume of chloroform, sealed well and left at 4°C for 12 hours. An aliquot of the chloroform layer is appropriately diluted with ethanol and absorbancies are read at 280, 330, 380 and 450 nm in a spectrophotometer. By use of a correction formula, the concentrations of vitamin A expressed as  $\mu\text{g}$  retinol/g wet weight and of carotenoids in  $\mu\text{g}/\text{g}$  are calculated. If desired, another aliquot of the chloroform phase can be directly analyzed colorimetrically at 620 nm by use of trichloroacetic or trifluoroacetic acid.

### Apparatus

Balance  
Ultraviolet-visible spectrophotometer  
Eppendorf or similar fixed volume syringe-pipette  
Micropipettes  
Screw top 50 ml bottles

### Reagents

Anhydrous sodium sulfate, reagent grade  
Chloroform, reagent grade, stabilized with 0.75% ethanol  
Absolute ethanol, reagent grade  
All-*trans* retinyl acetate, crystalline  
Trichloroacetic acid, reagent grade, white and dry crystals; alternatively, trifluoroacetic acid can be used.

### Procedure

#### *Extraction and Analysis*

Weigh 5 g liver to the nearest 0.1 g, place it in a 50 ml bottle with a screw top, and add 12.5 g anhydrous sodium sulfate. Gently mash with a large spatula to a viscous paste and cover with 25.0 ml chloroform. Swirl gently, seal tightly with a screw cap and place in a refrigerator overnight (8-24 hours). At the end of the extraction period the chloroform will form a clear layer over the caked residue.

Withdraw carefully 0.30 ml of the clear chloroform phase with a syringe-pipette and transfer it to 2.7 ml ethanol in a 1 cm cuvette. Read the absorbancies at 280, 330, 380 and 450 nm.

If the colorimetric reaction is also to be run, transfer 0.20 ml of the chloroform extract to a 1 cm cuvette, quickly squirt in 1.8 ml of freshly prepared 30% trichloroacetic acid (or 40% trifluoroacetic acid) in chloroform at ambient temperature, and read the maximum absorbancy at 620 nm, which usually occurs within 10 seconds. If the absorbance is very low (<0.06) and does not decrease over 20-30 seconds, the vitamin A content is taken as zero. Run at least one standard retinyl acetate assay (2  $\mu\text{g}$  retinol in the test) with each group of samples.

### Calculation

In the spectrophotometric assay, the corrected absorbancy at 330 nm is calculated by the formula:

$$\text{Corrected } A_{330} = 0.5(2.27 \times A_{330} + 0.17 \times A_{450} - A_{280} - A_{380}).$$

Then,

$$\mu\text{g retinol/g liver} = \frac{\text{Corrected } A_{330} \times \text{dilution factor}}{0.1795 \times \text{sample weight (g)}}$$

The  $E_{1\text{cm}}^{1\%}$  value of retinol (1850) is corrected to 1795 as a result of the 3% decrease in the absorbancy of retinyl esters in ethanol.

The dilution factor in the above case is 250. Furthermore,

$$\mu\text{g carotenoids/g} = \frac{A_{450} \times \text{dilution factor}}{0.238 \times \text{sample weight (g)}}$$

In the colorimetric assay, the amount of retinol in the test is determined from a standard curve based on at least five points (0.5, 1, 2, 3 and 4  $\mu\text{g}$  retinol in 2 ml of test solution).

Then,

$$\mu\text{g retinol/g liver} = \frac{\mu\text{g retinol in test} \times \text{dilution factor}}{\text{sample weight (g)}},$$

or 250 in the cited case.

When a significant amount of carotenoids is present in the liver extract, the following formula is used:

$$\text{Corrected retinol } (\mu\text{g/g}) = \text{Observed retinol } (\mu\text{g/g}) - \frac{\text{Carotenoids } (\mu\text{g/g})}{20}$$

The way in which the above formulas and constants were derived is carefully explained in the original paper.<sup>1</sup>

### Standards

The absorbancy of retinyl acetate in ethanol at 330 nm is linear with concentration to an absorbancy of at least 1.0. By using an  $E_{1\text{cm}}^{1\%}$  of retinyl acetate in ethanol at 330 nm of 1565, an absorbancy factor for retinyl ester, expressed in terms of retinol, of 1795 can be calculated, i.e.,  $1565 \times 328/286$ . In the colorimetric assay, a slight downward curvature was noted with increasing concentrations of retinol above 1  $\mu\text{g/ml}$ . In most cases the  $E_{1\text{cm}}^{1\%}$

at 620 nm for the transient blue complex in chloroform was  $4400 \pm 3\%$  under our assay conditions, about 12% below the reported maximum. An  $E_{1\%}^{1\text{cm}}$  value for beta-carotene in ethanol at 450 nm of 2375 is used in calculating the carotenoid content.

### *Reproducibility*

The reproducibility of the spectrophotometric assay is dependent on the concentration of vitamin A in the liver and the heterogeneity of its distribution. When concentrations are high ( $>80 \mu\text{g/g}$ ) and heterogeneity is minimized by mincing the liver before sampling, a C.V. of  $<5\%$  is readily achievable. When vitamin A concentrations are low ( $<10 \mu\text{g/g}$ ) and heterogeneity is uncontrolled, a C.V. of 30% is often found. When 1 g samples of liver are used, a C.V. of 50-60% is not unusual.

### *Sensitivity*

Vitamin A concentrations as low as  $1 \mu\text{g/g}$  liver can be determined.

### **Effect of Sample Storage**

Unhomogenized liver samples frozen at  $-20^\circ\text{C}$  are stable for months.

### **Variations and Modifications**

Sensitivity can be increased by reducing the volume of chloroform used as the extractant, increasing the aliquot size or adapting the method for fluorescence assay, which is much more sensitive.

### **Comments and Precautions**

- The method requires approximately 10 minutes for the preparation of each extract and an average of 20 minutes for conducting both analyses.
- When fresh samples of human liver are used, gloves should be worn. Frozen liver samples should be weighed quickly, and all materials in contact with the sample should be decontaminated in ethanol before disposal.
- Mashing should continue only to a point at which the sodium sulfate is *reasonably well mixed* with the liver paste. The mixture should *not* be ground to a fine powder because: (a) some of the fine powder may escape as an aerosol and thereby pose a health hazard, and (b) the chloroform layer becomes cloudy with fine particles, which adversely affect the assay and are difficult to remove. Ideally, the liver and sodium sulfate should cake together in the bottom of the vial during extraction with a crystal clear chloroform layer above it.
- Manual pipettes are used for all transfers, never suction by mouth.
- Fresh solutions of trichloroacetic acid are warmed to room temperature before use inasmuch as colorimetric readings are about 50% higher with the cold reagent.
- Some batches of trichloroacetic acid, even when dry, white and crystalline, do not give good color yields. The reason for the inactivity is not understood.

- If color yields are adequate for a given assay condition, even when  $E_{1\text{cm}}^{1\%}$  values at 620 nm are markedly lower than 4400, the reagent can, of course, be used. If not, another batch should be tried.

- The presence of water or ethanol markedly reduces the color yield in the colorimetric test. The small amount of absolute ethanol used to stabilize chloroform has not proven to be a problem, however.

## Laboratory Quality Control

Each method has its own intrinsic characteristics of reliability that depend on the chemical, physicochemical or biological basis of the assay, and determine the optimal performance under the best operational conditions. For any method, the two major aspects of reliability to be evaluated are:

- *accuracy*—conformity of the results with the absolute true value, and
- *precision*—the reproducibility of values by the method under the best operational conditions.

The present chapter is not concerned with the intrinsic reliability of each method *per se*, but with the systematic control of the quality of routine laboratory operations. Any analytical procedure is subject to a number of possible errors derived from the practical conditions under which the operation is performed. These include the “human factor” (efficiency of the technician), as well as factors such as room temperature, humidity, light exposure, and failure in equipment performance.

### Quality of the Procedure as Initially Set Up in the Laboratory

#### Accuracy

In setting up a method, each laboratory should confirm the intrinsic accuracy of the procedure, as originally proposed in the literature. The specific procedure for checking accuracy will vary from one method to another. In all methods, however, the recovery of known amounts of standard vitamin A added to the biological sample should be checked. Samples initially containing a “low,” “medium” and “high” concentration of vitamin A should be included in the testing.

#### Precision

Although most published methods include an indication of its precision, the coefficient of variation will obviously vary as a function of the exact procedure employed. Therefore, the reported precision of a given method should also be confirmed in each laboratory. Two types of measurements are usually made, within-run and between-run reproducibility.

## Within-Run Reproducibility

When a new method is initially set up, or at any time that one or more of its conditions are altered, the precision of the method should be evaluated. Alteration in conditions may include a new technician, new equipment or switching from macro to micro scale.

A relatively large amount of a control specimen should be available, both for within-run and between-run tests of reproducibility. In the case of serum, pooled human sera or pooled bovine sera usually serve this purpose well. Multiple aliquots of this homogeneous control material are then prepared according to the operational protocol of the specific method. The same measurement is made on replicates ( $N \geq 10$ ) during the same laboratory run, using the conditions that are planned for routine laboratory work. Ideally, aliquots should be dispensed into a series of individual test tubes as if they were different specimens. Preferably, three different concentrations of the substance within the expected biological range should be analyzed ("low", "medium" and "high"). If only one concentration level is selected, a relatively "low" level should be given preference, since in most surveys low levels are the biologically critical values.

If possible, 20 replicates should be prepared for the single run, although 10 is an acceptable minimum number. From the replicate determinations made, a mean (or average) and standard deviation are calculated for each run.

The following equation can be used:

$$\text{S.D.} = \sqrt{\frac{\sum x^2}{N - 1}}$$

where  $N$  is the number of determinations,  $\sum$  is the sum of, and  $x$  is the deviation of each determination from the mean.

Normally, in the process of setting up a method, either anew or after introducing a new factor (new technician, new equipment, etc.), the supervisor should have repeated analyses made until a plateau value, which agrees with the reported reproducibility of the method, is attained. As mentioned above, within-run reproducibility is expressed in terms of the standard deviation and coefficient of variation ( $\text{C.V.} = \frac{\text{S.D.}}{\text{mean}} \times 100$ ).

## Control of the Quality of the Operation Through Time (Between-Run Reproducibility)

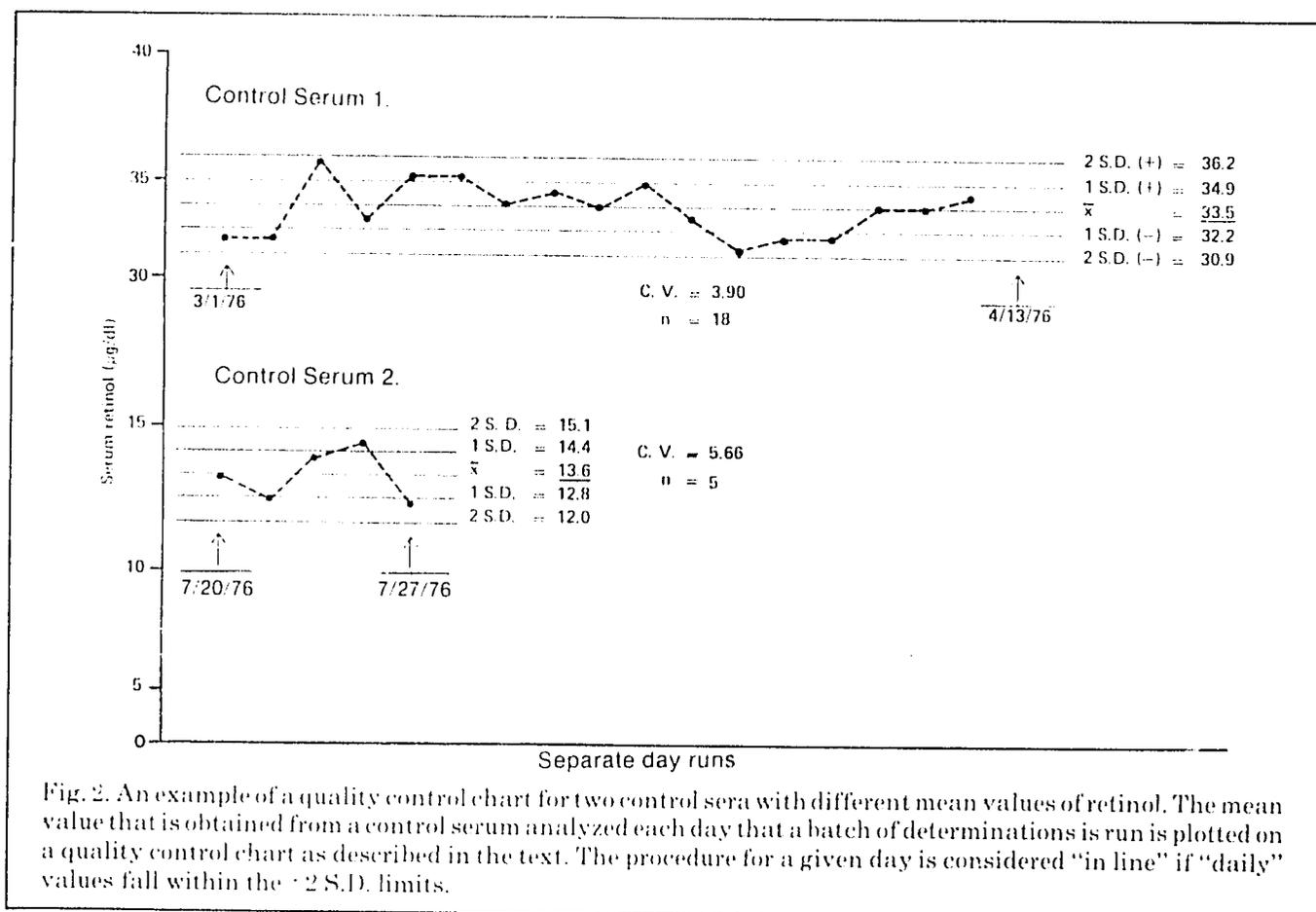
This section is devoted to discussing and recommending a system to determine when a method as applied routinely in a laboratory is "in line" or "out of line."

Once the mean values and standard deviations for the control specimens have been calculated, the data are used to determine the allowable error among day-to-day replicates in the routine runs.

Experience dictates that this allowable error should be conveniently set at  $\pm 2$  standard deviations (tolerance), since 95 percent of an infinite number of replicate values should fall within these set limits.

Quality control charts are then prepared by plotting the control data on the ordinate ( $y$  axis) of linear graph paper and time on the abscissa ( $x$  axis). The mean value and values falling 2 standard deviations above and below the mean are drawn as lines parallel to the  $x$  axis (Fig. 2).

Duplicate aliquots of the same control specimens that are used to determine the within-run variability are then introduced into the "daily" laboratory runs\* and the values are placed as points over the control chart described above. This provides the laboratory supervisor with a means of detecting at a glance whether the procedure is "in line", that is when the results of the control "daily" determinations fall within the  $\pm 2$  standard deviation limits. When they do not, the procedure is "out of line" and the reasons for the erroneous result ought to be determined. It should be noted that this procedure also gives early warning of possible methodological trouble in terms of observable trends within the "in line" region of the control chart (Fig. 2).



## Duplicate vs. Single Aliquots

As a general rule all determinations should be run in duplicate. This is aimed at increasing the reliability of the measurements. Under certain limiting circumstances, such as large field survey projects in which large population samples are combined with limited resources, a compromise may be accepted as follows: samples will be run in single aliquots, but a proportion of them (1:10 or 1:20, for example) will be run in duplicate; the number of specimens run in duplicate should be at least 10 and should be evenly interspersed among the specimens included in the complete set of samples comprising the runs.

Under ideal circumstances these duplicates should be "blind", that is, with only the supervisor, not the technician, knowing that they are duplicates. However, this

\*These specimen aliquots are to be preserved under the most strict conditions to ensure the stability of the substance to be measured (or lack of change), and only for as long as this stability (constancy of true value) can be assured. These conditions-time requirements are most commonly characteristic of the substance and the method.

requirement may not always be feasible, and it may even be possible to waive it when the operational reproducibility of the method has been shown to be high. Testing for this can be done as illustrated by the actual example shown in Table 4.

A total of 36 separate aliquots of a control serum were analyzed individually for retinol by the spectrophotometric method of Bessey *et al.* (p. 19). The individual values are listed in the table, together with estimates of reproducibility in three different ways:

- The 36 samples were paired to constitute 18 “duplicate specimens” simulating the situation in a laboratory where all determinations are always run in duplicate.
- The 36 individual aliquots were divided into two sets of 18 individual specimens. Arbitrarily, the first 18 determinations were considered to be the first set and the second 18 determinations were analyzed as a second set.
- The 36 values were taken as individual specimens, but 18 selected at random, were termed the “first random set,” whereas the rest (N=18) constituted “the second random set.”

TABLE 4.  
Variability of a Control Serum Through a Series of Runs of Serum Retinol

Date	$\mu\text{g./dl}$		
3-01-76	31.2		
	32.5		
3-02-76	31.8		
	32.5		
3-05-76	35.0		
	35.7		
3-09-76	33.0		(a) Duplicates
	33.0		N = 18
3-11-76	35.0		$\bar{x}$ = 33.6
	34.4		S.D. = 1.31
3-12-76	35.8		C.V. = 3.90
	34.4		
3-16-76	33.0	(b) First set	(b) Second set
	33.6	N = 18	N = 18
3-18-76	33.8	$\bar{x}$ = 33.6	$\bar{x}$ = 33.3
	33.0	S.D. = 1.32	S.D. = 1.32
3-19-76	32.2	C.V. = 3.94	C.V. = 3.95
	34.2		
3-22-76	35.0		
	34.2		
3-23-76	34.2	(c) First random set	(c) Second random set
	31.5	N = 18	N = 18
3-24-76	32.2	$\bar{x}$ = 33.2	$\bar{x}$ = 33.6
	30.8	S.D. = 1.23	S.D. = 1.39
3-26-76	32.2	C.V. = 3.69	C.V. = 4.15
	32.8		
3-29-76	31.5		
	32.2		
3-30-76	33.6		
	34.2		
3-31-76	32.8		
	34.2		
4-01-76	34.2		
	34.2		
4-13-76	34.2		
	35.0		

The significance of the differences between the three approaches can be statistically tested. In the example, it is evident that with the degree of precision attained in this laboratory run, the use of duplicate determinations for all the samples to be assayed did not add to the quality of the data. However, inclusion of some check duplicates is still essential for the control of method precision.

### **Use of the Duplicate Data**

When all the specimens are run in duplicate, it is generally recommended not to accept duplicate values differing by more than  $\pm 2$  standard deviations from the mean. The value of this S.D. is obtained through the series of tests of the precision of the method as described earlier in this chapter. In the case of sample rejection, a completely new determination should be run; if this is not possible, the sample should be discarded. The application of the concept of "blind" (independent) duplicates may not be feasible under routine conditions. In this case the supervisor has to exert extreme vigilance over the technician in order to minimize the known bias in the error estimation.

In the case of duplicates in a sub-sample only, the purpose would be to have an estimate of error, since there is no use in repeating the aliquots in only a few specimens. A good practical rule is that if 95% of the duplicates differ by less than  $\pm 2$  S.D., it may be concluded that the method is under control in this respect. Again, it is to be recognized that if the duplicates are not independent, some degree of over estimation of the true precision may occur.

### **The Key Role of the Laboratory Supervisor and the Analyst in Quality Control**

It is essential that both the laboratory supervisor and the analyst-technician be thoroughly aware of their responsibility to adhere most strictly to methodological instructions. Any deviation from such instructions cannot be introduced without the laboratory director's approval.

In addition, the training of the laboratory personnel must include a strong motivational component aimed at realizing and accepting the "professional" commitment to registering and reporting the data, and in fact any datum, as read. This means a special individual effort to avoid, for instance, the influence of the value read for the first tube of a duplicate specimen, on the second. The tendency to do this is not necessarily related to "personal honesty," but is a natural phenomenon in all individuals that can be statistically estimated.

## CHAPTER VII

# Data Analysis

### References

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## Introduction

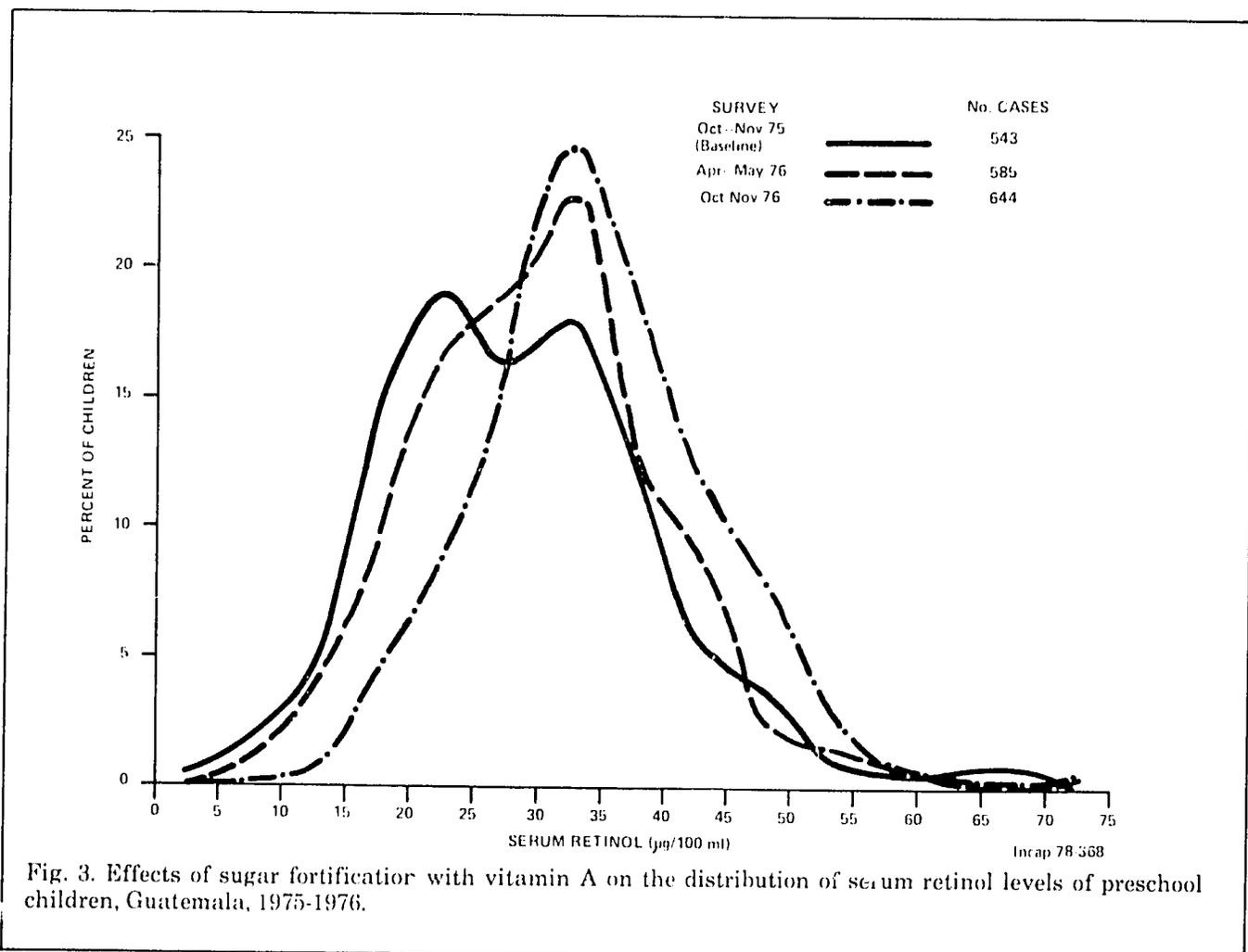
Biochemical measurements of the vitamin A status of populations are generally used either:

- to compare the levels of retinol, or of some vitamin A-related substance, between two groups, e.g., a known, well-nourished control group and a group which may be at risk, or
- to evaluate the effect of an intervention program or natural changes in status with time, e.g., a before and after comparison.

Appropriate ways of presenting and analyzing comparative vitamin A data from such surveys will first be considered. Thereafter, some guidelines for interpreting vitamin A data relative to the nutritional status of a given population will be presented. Finally, the interpretation of plasma RBP and carotenoid values will briefly be discussed.

### Distribution of Biochemical Values in a Population

Values in populations are not always distributed in a normal fashion. The form of the distribution curve should therefore be determined by plotting the biochemical values along the abscissa (x axis) and its frequency on the ordinate (y axis). In constructing the plot, biochemical values are grouped into convenient ranges of equal size. Grouping is a critical activity: if too many groups are selected, the sample number found in each range



is small and the nutritional significance of adjacent groups may be obscure. If too few groups are used, valuable nutritional information may be lost. Usually, the selection of 10-20 groups optimizes use of the data. The application of this method to serum retinol values obtained in the evaluation of a sugar fortification program, in which 15 groups at 5  $\mu\text{g}$  retinol/dl intervals were selected, is given in Fig. 3.

The mode, range of values (individual variability), and general shape of the distribution in terms of symmetry, skewness or other characteristics (e.g., bimodal and log normal distributions) are readily apparent in this type of plot. When they appear, bimodal curves can be analyzed as overlapping monomodal curves. Log normal distributions can be analyzed more effectively by conventional statistical tests after normalization of the distribution by logarithmic transformation.

For normal or near-normal distributions the data can be summarized using the mean and the standard deviation. On the other hand, skewed distributions are more appropriately expressed in terms of the median and percentile values. An example of the use of percentiles to assess the impact of a nutritional intervention program<sup>1</sup> over an 18-month period is given in Table 5.

Period	No.	Percentile Values ( $\mu\text{g}$ $\mu\text{g}$ liver tissue)				
		5th	10th	50th	90th	95th
Before fortification	80	16	29	103	292	320
After fortification	123	75	96	203	463	537

This type of presentation permits some important conclusions to be made about the data: (1) that the distribution of liver concentrations is highly skewed, e.g., the difference in vitamin A concentrations between the median (50th percentile) and the 95th percentile is two to three times greater than that between the median and the 5th percentile, and (2) that the largest proportional increases in vitamin A concentrations as a result of the intervention occur in the lower percentile ranges, e.g., the 5th percentile increased nearly fivefold while the 95th percentile increased less than twofold.

The practical usefulness of this type of data presentation for comparing two situations is clear: the statistical significance of the differences observed can be tested by conventional statistical methods.

An alternative form of graphic presentation of distributions is the histogram (Figs. 4 and 5). When the number of value-classes is less than 10 due to the smallness of the sample size, this method may be the appropriate choice.

### Use of Specific Guidelines

Quite apart from the presentation of all the data obtained in tabular or figure form, specific guidelines may be applied. These guidelines usually connote degrees of nutritional adequacy or risk.

The ICNND guidelines for serum retinol<sup>1</sup> is an example of this method, in which cutoff points for so-called "deficient", "low", "acceptable" or "high" categories are specified. The distribution cutoff points for the categories, defined on the basis of biological and epidemiological considerations, permits the assignment of a certain degree of increasing

nutritional inadequacy or risk to values that fall below a chosen cutoff point. Although these cutoff points are relatively arbitrary, they reflect the best judgment of investigators experienced in nutritional appraisal. The designations “deficient”, “high risk”, “low risk” or “moderate risk”, etc., are self-explanatory. On the other hand, they do not connote any expected frequency of clinical alterations in a given category. Furthermore, cutoff points established for populations are not intended for use with individuals.

In considering the extent of a nutritional problem in a population, the next logical step is to establish prevalence values above which a public health problem of unacceptable magnitude exists, i.e., 5% or more of preschool children with serum retinol values  $<10 \mu\text{g}/\text{dl}$  (the “deficient” category). Like cutoff points, these prevalence criteria, although arbitrary, are based on the best judgment of expert nutritionists familiar with survey methods and results.

Despite their limitations, guidelines and prevalence criteria have proved helpful to public health nutritionists and policymakers by calling attention to the undesirable status of a certain population group with respect to a particular nutrient. Most importantly, they have facilitated the establishment of goals in planning, implementing and evaluating intervention programs. However, they should not be applied indiscriminately or in isolation when evaluating the vitamin A status of individuals.

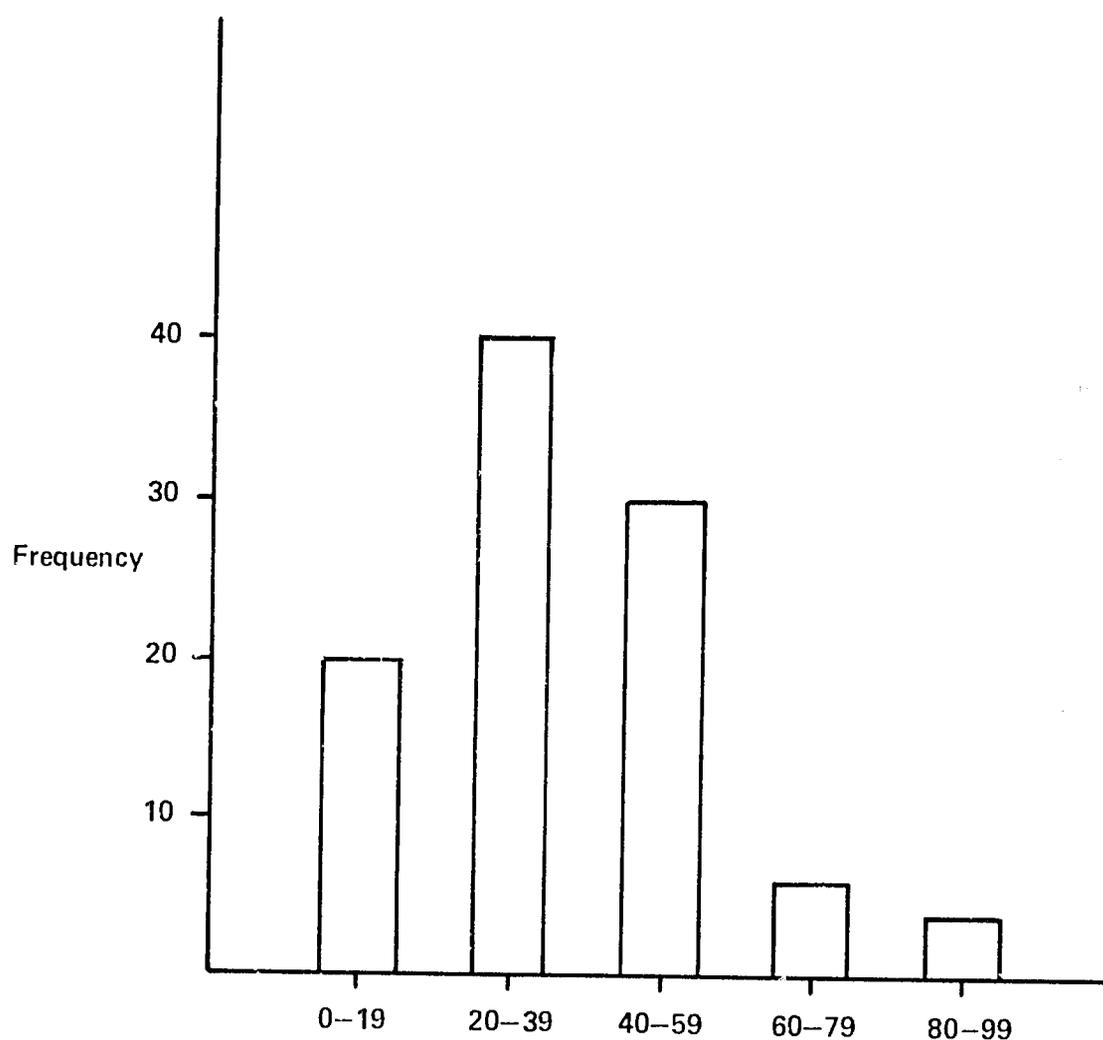


Fig. 4. A possible distribution of breast milk vitamin A in rural women in Central America.

# Biochemical Guidelines and Prevalence Criteria for Vitamin A

As mentioned earlier, guidelines and prevalence criteria are arbitrary but useful indices for interpreting survey data. In the present section, indices will be suggested for vitamin A in serum, milk and liver. Whereas vitamin A in serum is a widely used indicator of the vitamin A status of populations, milk and liver values have not been generally used for this purpose. The guidelines suggested for these latter two indices are consequently tentative. Continued efforts should be made both to improve existing indices and to establish new ones as our knowledge of means to assess vitamin A status increases.

## Serum Vitamin A

The guidelines developed for serum vitamin A assume that blood is collected under fasting conditions. Inasmuch as a breakfast may have little effect on serum values, specimens from nonfasting subjects are usually acceptable. In any case, the collection conditions should be specified in the survey report. Serum vitamin A, because of the homeostatic control exerted by the liver, is not a good general indicator of total body stores.<sup>2,4</sup> Nonetheless, low serum vitamin A values are suggestive of a depleted state, with or without the presence of clinical signs of deficiency.<sup>1,3,4</sup>

Based on the Sheffield experiment in England,<sup>5</sup> the following guidelines for serum retinol were set by the ICNND in 1963:

Deficient,  $<10 \mu\text{g}/\text{dl}$ ; Low,  $10\text{-}19 \mu\text{g}/\text{dl}$ ; Acceptable,  $20\text{-}49 \mu\text{g}/\text{dl}$ ; High,  $>50 \mu\text{g}/\text{dl}$ .

Later, the following prevalence criteria for the existence of a public health problem were established:

$\geq 5\%$  of a selected group with serum retinol values  $<10 \mu\text{g}/\text{dl}$ , or  $15\%$  with values  $<20 \mu\text{g}/\text{dl}$ .

These old ICNND guidelines,<sup>4</sup> although useful at the time, had several defects:

- they were drawn up on the basis of studying only a few adults;
- they are not generally applicable to children, who, as pointed out below, have lower plasma vitamin A values;
- the defined high range has little nutritional or physiological significance; indeed the range for a normal well-nourished population often extends well beyond  $50 \mu\text{g}$  retinol/dl.

These points will be discussed in more detail below.

Inasmuch as *very* low serum values are highly indicative of a depleted state and are often associated with clinical signs of vitamin A deficiency, the deficient category ( $<10 \mu\text{g}/\text{dl}$ ) and the prevalence criterion for that group ( $\geq 5\%$ ) were adopted by the World Health Organization as a recommended biochemical indicator of a vitamin A deficiency problem in a population.

Higher serum values ( $>10 \mu\text{g}/\text{dl}$ ) are much more difficult to interpret. Among adequately nourished healthy children an age-related rise occurs early in life<sup>2</sup> and the distribution of mean values before puberty is lower ( $30\text{-}40 \mu\text{g}/\text{dl}$ ) than after puberty ( $45\text{-}60 \mu\text{g}/\text{dl}$ ) (Fig. 5). In general, adult women before menopause have mean levels slightly lower than males, but of the same magnitude after menopause.

As noted earlier, the liver acts as a type of regulator over plasma levels of vitamin A for individuals. Thus, the regulatory mechanism at levels of vitamin A nutriture above the deficiency state is only partially influenced by the vitamin A level in the diet. Other factors, such as environmental stress, chronic parasitism, acute and chronic infections, and probably a genetic component, influence the level of circulating vitamin A. Fluctuating steroid hormone levels, whether natural or due to the ingestion of oral contraceptives or medication, also influence serum vitamin A. There may be additional factors as yet unrecognized because of our incomplete understanding of the liver's handling of the vitamin and its transport protein under a variety of environmental and physiological conditions.

Consequently, the setting of an "adequate" or "acceptable" range for serum vitamin A which is universally applicable poses many problems. Since few manifestations of vitamin A deficiency are evident in individuals with serum values of 20  $\mu\text{g}/\text{dl}$  or more, this level has been proposed as a cutoff for the "adequate" category. This guideline is much less firmly established than the "deficiency" category, however, and when applied to young children may overestimate the magnitude of the problem. Nonetheless, the so-called "adequate" and "deficient" categories have been used effectively in showing the efficacy of a vitamin A intervention program.<sup>14</sup>

Until more data become available, it is safer to adhere to this single guideline (20  $\mu\text{g}/\text{dl}$ ) as the cutoff for acceptable serum levels without age differentiation. It is to be emphasized, however, that when possible the distribution of vitamin A values in young children should be reported separately from whole population data.

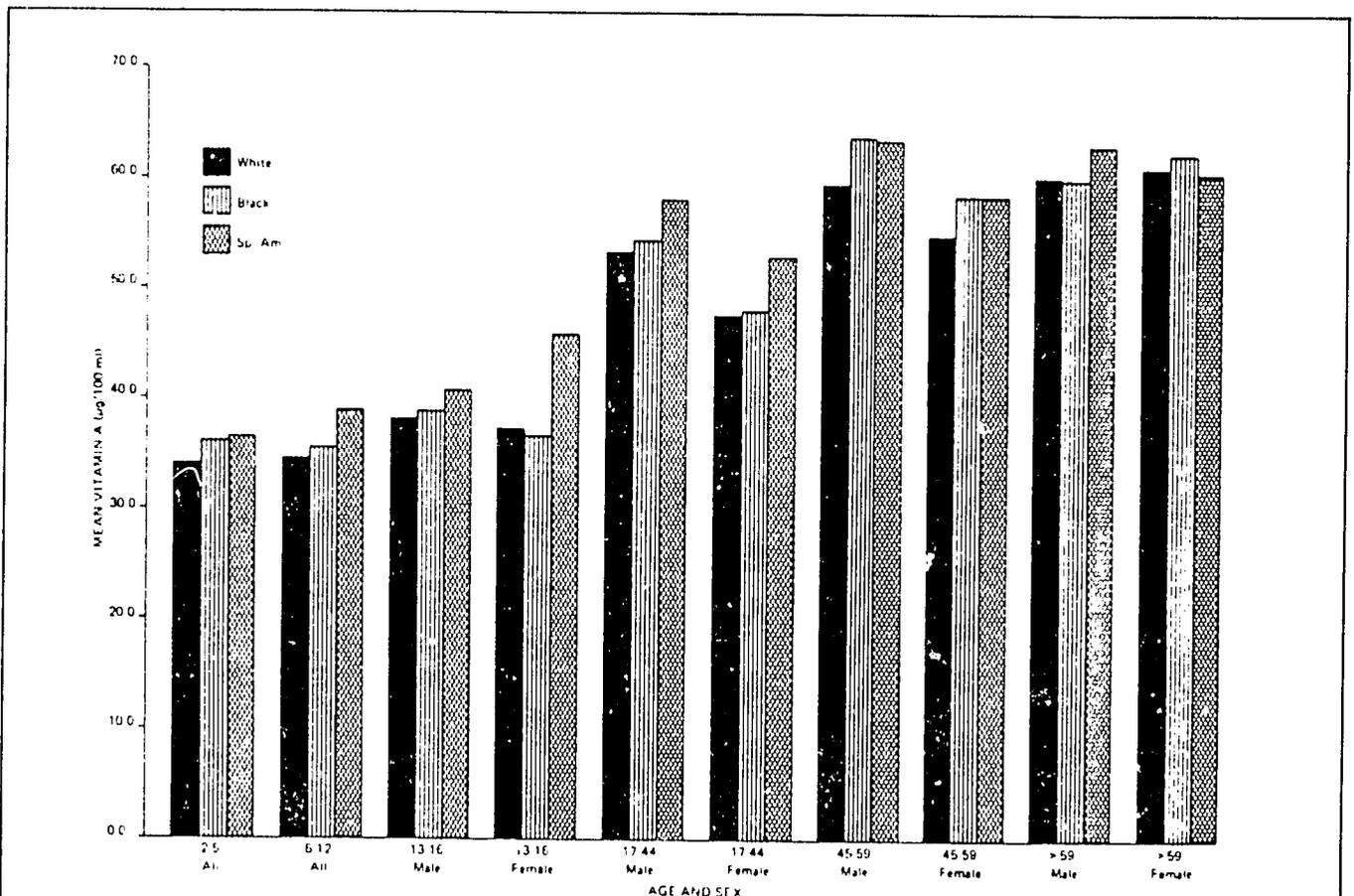


Fig. 5. Mean plasma vitamin A values by age, sex and ethnic group for high income ratio states—Ten-state nutrition survey (1968-1970), figure 1b, IV-139.

## Breast Milk Vitamin A

The concentration of vitamin A in breast milk may also contribute to the assessment of vitamin A nutritional status in population surveys.<sup>1</sup> In populations with generally inadequate dietary intakes of vitamin A, lactating women secrete milk with a reduced content of vitamin A. Since vitamin A in the milk is derived both from holo-RBP and from lipoprotein-bound retinyl ester in the mother's blood, a decreased concentration of vitamin A in the milk reflects both the mother's poor vitamin A intake and her inadequate body stores. When women consume sugar fortified with about 10  $\mu\text{g}$  vitamin A/g instead of unfortified sugar during their last trimester of pregnancy and the first four months of lactation, the vitamin A content of their breast milk increases and the serum retinol levels of both mother and nursing infant rise.<sup>6</sup>

Apart from the influence of the nutritional state of the lactating mother on milk vitamin A values, human milk can also serve as a useful indirect indicator of the likely vitamin A nutritional status of breast-fed infants. In many developing countries, breast-fed infants receive insignificant amounts of additional vitamin A in their complementary foods. Under these conditions, breast milk provides essentially all of the vitamin A ingested.

The average vitamin A content of breast milk from well-nourished lactating women is around 50  $\mu\text{g}/\text{dl}$ . This value, together with an estimated daily milk volume of 850 ml, has been used to estimate the recommended dietary allowance of vitamin A for infants.<sup>7</sup> In many situations, however, both the vitamin A concentration and the daily milk volume are much lower, particularly in malnourished women. The establishment of a tentative guideline for milk vitamin A concentration would therefore be helpful both in assessing maternal vitamin A status and in evaluating intervention techniques.

It is consequently proposed that a vitamin A level of 20  $\mu\text{g}$  retinol/dl of milk, obtained as described on page 9 from a full breast, be taken as the cutoff point below which the vitamin A intake of both mother and breast-fed infant are likely to put them at risk of vitamin A deficiency. It is further proposed that a prevalence of 15% or higher of values below 20  $\mu\text{g}/\text{dl}$  be considered inadequate from the public health standpoint, calling for an intervention program to improve the vitamin A status. Together with other indices, this guideline has been used in evaluating the national vitamin A sugar fortification program in Guatemala.<sup>1</sup> After only 18 months of sugar fortification, the prevalence of breast milk samples with less than 20  $\mu\text{g}/\text{dl}$  fell from an unacceptable baseline value of 39% to only 11%.

## Liver Vitamin A

Since most of the total body stores of vitamin A ( $\geq 90\%$ ) are stored in the liver, direct measurement of liver vitamin A concentrations gives information about vitamin A status not provided by other biochemical or clinical indices. The only way to obtain such information at present is by the analysis of liver specimens obtained at autopsy. Although of limited use in the overall assessment of the vitamin A status of a population, extrapolation of such autopsy data to selected living populations has been made.<sup>8,9</sup> Liver data have also been effectively used in assessing the efficacy of a sugar fortification program.<sup>1</sup>

The criteria of "adequacy" or of critically "low values" are based essentially on the concept of the "protection period", which is defined as the period for which a given total body store of vitamin A will satisfy all metabolic needs in a child on a vitamin A-free

diet.<sup>10</sup> The suggested length of the protection period is 100 days (about 3 months), inasmuch as this period would bridge the seasonal scarcity of fresh vitamin A and carotenoid-containing foods, acute childhood diseases, episodes of anorexia and the like. To maintain adequate vitamin A status for 100 days, a liver reserve of 20  $\mu\text{g/g}$  has been estimated to be sufficient on the basis of metabolic, turnover and excretion data.

It is, therefore, proposed that a level of liver reserves of vitamin A equivalent to 20  $\mu\text{g}$  retinol/g fresh wet liver be considered as the cutoff point for acceptable hepatic reserves. It is also proposed that a liver vitamin A concentration of 5  $\mu\text{g/g}$  be set as a cutoff point below which the risk of clinical vitamin A deficiency is deemed to be very high. In terms of prevalence criteria, a problem of significant public health concern is considered to exist when the prevalence of hepatic retinol values below 5  $\mu\text{g/g}$  in the preschool age group is  $\geq 3\%$ , and/or the prevalence of values  $< 20 \mu\text{g/g}$  in the same group is  $\geq 15\%$ . Suggested guidelines for interpreting vitamin A status are summarized in Table 6.

TABLE 6.  
Biochemical Criteria for the Assessment of Vitamin A Status<sup>a</sup>

Relative Status	Plasma $\mu\text{g/dl}$	Breast Milk $\mu\text{g/dl}$	Liver $\mu\text{g/dl}$	Expected Dietary Intake <sup>b</sup> $\mu\text{g/dl}$
Well-nourished	$> 30$	$> 50$	$> 50$	$\geq 750$
Adequate	$> 20$	$> 20$	$> 20$	$\geq 400$
Critical	$< 10$	NK	$< 5$	$< 100$
Prevalence limit	5% $< 10$ 15% $< 20$	NK 15% $< 20$	3% $< 5$ 15% $< 20$	NK NK

<sup>a</sup>All values are expressed as  $\mu\text{g}$  retinol

<sup>b</sup>Expressed as  $\mu\text{g}$  retinol equivalents, where 1  $\mu\text{g}$  retinol = 6  $\mu\text{g}$  beta-carotene = 12  $\mu\text{g}$  mixed food carotenoids

<sup>c</sup>The population considered is defined in the text

NK = Not known

## Interpretation of Data Relating to Plasma Retinol-Binding Protein and Serum Carotenoids

The most useful indices of vitamin A status quite naturally concern vitamin A itself. Nonetheless, two ancillary measurements, plasma retinol-binding protein and serum carotenoids, do provide additional specialized information. The measurement and interpretation of RBP is considered in detail beginning on page 44, and information on carotenoid analysis is presented in Chapter IX. Nonetheless, some comments about the interpretation of each of these ancillary measurements might be made.

Holo-RBP analysis is useful because it alone provides a measure of what current knowledge indicates is the physiologically active form of vitamin A in the circulation. In malnourished children treated therapeutically with a complete diet including, of course, vitamin A and protein, the increase in plasma holo-RBP serves as a useful indicator of recovery.

In normal adults the range of total RBP concentrations is from 4-9.0 mg/dl. In normal preschool children values are generally within the range of 2.5-3.5 mg/dl. Most ( $> 80\%$ ) RBP in well-nourished persons circulates as holo-RBP. In protein- and vitamin A-deficient children, however, the RBP concentration falls to 50% or less of the normal value, and in severe cases RBP may be completely in the apo-form, i.e., retinol will be virtually absent from the plasma. Thus, caution is needed when interpreting values of total RBP as

indicative of circulating levels of retinol under conditions of severe protein and/or vitamin A deficiency. When it is not possible to determine holo-RBP (pages 50 and 53) in populations at high risk of deficiency, serum vitamin A values alone should be employed.

In regard to plasma carotenoids, the ICNND<sup>1</sup> first proposed empirical range categories for interpreting "carotene" values in blood plasma, namely high,  $>100\mu\text{g}/\text{dl}$ ; acceptable,  $40\text{-}99\mu\text{g}/\text{dl}$ ; and low,  $<39\mu\text{g}/\text{dl}$ . "Carotene" was operationally defined in terms of the absorbance at 460 nm of pigments extracted from plasma by an appropriate organic solvent. Values of blood plasma carotenoids obtained by simply measuring light absorbance at 460 nm clearly are not by themselves interpretable in terms of vitamin A nutriture.

On the other hand, when used in association with plasma vitamin A values, plasma carotenoid values can provide some insight into dietary patterns of individuals or populations. Some possible relationships between plasma values of carotenoids and vitamin A are considered below:

- Plasma values of both carotenoids and vitamin A are in the adequate or high ranges. This plasma picture is normally to be expected in populations consuming liberal amounts of a mixed diet composed of animal foods and carotene-rich vegetables, fruits and oils. The latter might include green leafy vegetables, deep yellow- or orange-colored fruits and vegetables, and red palm oil. This blood profile is also found in populations living predominantly, or even strictly, on vegetable diets which contain abundant sources of provitamin A carotenoids.

The nature of the carotenoids in plasma will vary greatly, depending on the specific foods consumed. Normally, however, 10-50% of total circulating carotenoids consist of provitamin A molecules, among which beta-carotene usually predominates. In order to obtain more precise information about the composition of plasma carotenoids, components must be separated chromatographically and individually identified (Chapter IX).

- Plasma values of carotenoids are low, whereas plasma vitamin A values are adequate or high. This finding is commonly encountered in populations living on predominantly vitamin A-rich and carotene-poor diets (animal sources). If this relationship were found in subjects consuming the mixed animal-vegetable diet described above, it would suggest a condition of inefficient carotene absorption with normal intestinal utilization of vitamin A. The vitamin A nutritional status of these subjects ought to be diagnosed as satisfactory.

- Plasma values of both carotenoids and vitamin A are low. This plasma picture would naturally be found in populations consuming predominantly grains and/or other vegetables essentially devoid of carotenoids (both active or inactive). Such a finding, which is typical of poor populations in many less industrialized countries, would have to be interpreted as indicating hypovitaminosis A. If these plasma findings are encountered in subjects consuming diets rich in active carotenoids or rich in both carotenoids and retinol, a severe malabsorption syndrome is probably present.

- Plasma carotenoid values are apparently normal or high, whereas plasma vitamin A values are markedly depressed. Some populations, which may actually be suffering from hypovitaminosis A, use orange- or yellow-colored condiments, not of a carotenoid nature, to color or spice their foods. Some of these pigments are transported in the plasma, are extracted by organic solvents and absorb at 460 nm. Thus the presumed plasma carotenoid values are spurious. When such a situation is suspected, further

characterization of the pigments by chromatography, UV spectra and the like will clarify the nature of the colored compounds.

- Plasma values of carotenoids are greatly elevated, whereas plasma retinol levels are normal or high. This situation may be found in populations customarily ingesting large amounts of some very rich source of carotenoids (such as red palm oil), or occasionally in individuals on self-prescribed diets supplemented with abusive amounts of carotenoid-containing foods or formulas. These subjects often develop a yellow skin coloring (most noticeable on the palms of the hands and on the soles of the feet) which is clinically recognized as hypercarotenemia. This condition is benign, has no known adverse physiological effect, and disappears slowly upon reducing the excessive intakes of carotenoids.

In summary, total serum carotenoid values *per se* do not lend themselves to interpretation in terms of vitamin A nutritional status. When carotenoid data are analyzed together with information on dietary and serum retinol patterns of the population, however, they have complementary and/or confirmatory value for diagnosis of the nutritional situation. Although more rarely encountered, abnormalities in the biological utilization of sources of vitamin A activity in foods may also be detected.

## Interrelationships Among Indicators of Vitamin A Status

Various types of indicators have been used for estimating vitamin A status: (1) dietary intake of vitamin A and carotenoids; (2) biochemical measurements of vitamin A and carotenoids in fluids and tissues; (3) night blindness and other physiological symptoms caused by or associated with vitamin A deficiency; and (4) clinical signs of deficiency with particular attention given to the eye. Each class of indicator may provide some unique information about vitamin A status, but each taken alone has limitations in interpretation that are both inherent and technical. When data on populations using several indicators are all in accord, interpretation is straight forward relative to the state of vitamin A nutriture.

For example, in severe vitamin A deficiency, a good correlation exists among essentially all indicators: namely, specific clinical signs are present; the dietary intake of provitamin A and vitamin A can be documented as being very low in the preceding period; night blindness, when determinable with accuracy, is or has been present; plasma values of vitamin A, and of course *holo-RBP*, are very low; and the liver vitamin A, when determinable after the death of an affected child, is essentially depleted.

When vitamin A deficiency is less acute, however, various indicators obtained in cross sectional surveys correlate less well, thus clouding interpretation. For example, clinical eye signs may be present in children with plasma retinol values of 10-15  $\mu\text{g}/\text{dl}$ , some dietary intake of carotenoids and no clearly documented night blindness. Or, alternatively, plasma retinol values may be less than 10  $\mu\text{g}/\text{dl}$  in some individuals with no indication of either clinical eye signs or night blindness. These cases, which are common, should not lead us to reject some indicators as less reliable or less sensitive than others, but rather should remind us of the complexity of the physiological factors, in addition to diet, which influence each indicator separately from others. Among these factors are: balance among nutrients in the diet quite apart from carotenoids and vitamin A; the presence and severity of various acute and chronic diseases and parasitic infestation; and the rate at which signs of deficiency appear once the vitamin A reserve in the body falls to acute deficiency levels and disappear when conditions improve. All of these factors will affect the relationship among indicators, particularly when considering individual cases. This concept of the temporal relationship among indicators is depicted in Table 7.

When populations of suitable size are considered, correlations again become more useful for the mildly deficient and sub-optimally nourished groups. Thus, low dietary intake of carotenoids and vitamin A, the presence of clinical signs of deficiency usually associated with protein-energy malnutrition, and low plasma, liver and breast milk levels of vitamin A are often *statistically* correlated, and all respond in the expected fashion to a well-designed and well-implemented intervention program.

Of course, appropriate precautions must be exercised to adapt evaluation procedures and their interpretation to local circumstances. For example, the total intake of vitamin A-active substances in raw foods, as evaluated from food composition tables, may be misleading if the local practice of preparing or storing foods causes unusual losses of vitamin A, or if such foods are not fed to very young children. Similarly, the collection and handling of biological specimens, as well as their analysis, are assumed to be conducted properly

Finally, and recognizing the above caveats, three of the mentioned indicators—namely, liver and milk concentrations of vitamin A and dietary intake—not only can indicate an unacceptable state of vitamin A nutriture, but can be employed directly or indirectly as measures of good vitamin A status. Liver vitamin A concentrations from post-mortem sampling can be used to obtain a quantitative measure of total body stores; breast milk vitamin A concentrations, when combined with good estimates of daily milk volume, to yield a semi-quantitative indication of the daily intake of vitamin A by infants; and dietary data, particularly when foods rich in provitamin A and vitamin A are routinely ingested, to reassure us that the vitamin A status is satisfactory.

**TABLE 7.**  
Temporal Relationships Among Physiological Parameters and Clinical Signs  
at Onset and During Correction of Vitamin A Deficiency

Vitamin A-related Parameter	Deficiency		Recovery		Indicator	Assessment Method
	Change	Sequence <sup>a</sup>	Change	Sequence <sup>a</sup>		
Dietary intake	Chronically low	1	Increases	1	Diet	Food survey
Tissue reserves	Fall	2	Build up	6	Liver	Biochemical
Body fluid levels	Fall	3	Recover	3-4	Blood	Biochemical
					Breast milk	Biochemical
Intracellular levels	Fall	4	Increase	2	Cell content	None
Function	Declines	5	Recovers	3-4	Night blindness	Dark adaptation, interview
Clinical signs	Appear	6	Disappear	5	Xerophthalmia	Clinical
Permanent lesions	Appear	7	Remain	—	Keratomalacia, blindness	Clinical, functional

<sup>a</sup>The sequence indicates the order in which signs are expected to appear or disappear. Thus, during recovery, dietary intake increases first (1) followed by an increase in intracellular units (2), and so on.

## CHAPTER IX

# The Properties, Handling and Analysis of Carotenoids

### Introduction

The carotenoids represent the most widely distributed group of naturally occurring pigments found in nature. They are always found in photosynthetic tissue and are sporadically present from microbial life to man. Some 500 carotenoids have been identified, and of these, about 50 show provitamin A activity in animals.

Beta-carotene is an important pigment because of the color it imparts to fruits, vegetables and animal products. The visual and UV light absorption maxima of beta-carotene are due to its conjugated system of double bonds and its ring structure. Likewise, its provitamin A activity is due to its structural similarity to retinol. Substitutions in the basic structure, or changes in the double bond or ring structure generally result in lowered or eliminated provitamin A activity.

The carotenoids of food products are usually lipid-soluble, but may be complexed with protein or esterified. During the digestion process, the carotenoids are released and solubilized by the bile salts. Within the mucosal cell, beta-carotene is cleaved by dioxygenase(s) to yield two molecules of retinaldehyde, and other carotenoids are oxidatively cleaved to apo-carotenoids. Other carotenoids, depending on their structure, are absorbed with varying degrees of efficiency. Carotenoids are oxidized by some fish, birds and crustacea to pigments which are laid down in their skin, shell, eyes, milk, etc.

### General Precautions

### References

1. Davies, B. H. (1976) Carotenoids. In: *Chemistry and Biochemistry of Plant Pigments*. Vol. 2, T. W. Goodwin, ed., London: Academic Press.
2. Simpson, K. L., Lee, T-C., Rodriguez, D. B., and Chichester, C. O. (1976) Metabolism in senescent and stored tissues. In: *Chemistry and Biochemistry of Plant Pigments*. Vol. 2, T. W. Goodwin, ed., London: Academic Press.
3. Sauberlich, H. E., Dowdy, R. P., and Skala, J. H. (1972) *Laboratory Tests for the Assessment of Nutritional Status*. Cleveland, Ohio: Chemical Rubber Press.
4. Gebhardt, S. E., Elkins, E. R., and Humphrey, J. (1977) Comparison of two methods for determining vitamin A values of clingstone peaches. *J. Agric. Food Chem.* 25:628.
5. Zakaria, M., Simpson, K., Brown, P. R., and Krstulovic, A. (1979) Use of reversed-phase high-performance liquid chromatography analysis for the determination of provitamin A carotenes in tomatoes. *J. Chromatog.* 176:109.

Carotenoids are easily degraded with the production of artifacts.<sup>1,2</sup> Acids, particularly in the presence of light, cause the formation of *cis-trans* isomers with a shift in color. The actual breakdown of carotenoids is postulated to be through the formation of epoxides either at the 5,6 position or in-chain. Epoxide formation may be catalyzed by fluorescent light in the presence of oxygen and can be inhibited by free radical scavengers such as ethoxyquin. Lipoxygenase, if present in the tissue, could act on polyunsaturated fat with the carotenoids as secondary substrates. It has been observed that red fish become discolored at refrigeration temperatures in the dark. The product of lipoxygenase action on carotenoids has been characterized as colorless apo-carotenals. Some column chromatography stationary phases, such as magnesium oxide, Microcel C, silica gel and alumina, have been shown to degrade some carotenoids.<sup>3</sup> Generally, storage should be in the dark, under nitrogen at temperatures less than  $-20^{\circ}\text{C}$ . However, under these conditions some chemical and enzymatic changes still occur.

Significant error can result if beta-carotene is estimated by methods that do not separate the carotenoids. In a number of the vitamin A methods, the "carotene" fraction of plasma is estimated by its absorbancy at 450-460 nm or on the development of a secondary color. Non-provitamin A xanthophylls and carotenoids in plasma contribute to an overestimation of "beta-carotene." The degree of overestimation depends on the diet since blood carotenoids reflect dietary intake. The AOAC method used for food analysis can also overestimate the provitamin A content by significant amounts.<sup>1,3</sup>

## Extraction

### References

1. Davies, B. H. (1976) Carotenoids. In: *Chemistry and Biochemistry of Plant Pigments*. Vol. 2, T. W. Goodwin, ed., London: Academic Press.
2. DeRitter, E. and Purcell, A. E. (1981) Carotenoid analytical methods. In: *Carotenoids as Colorants and Vitamin A Precursors*. J. C. Bauernfeind, ed., New York: Academic Press.
3. Sweeney, J. P. and Marsh, A. C. (1973) Liver storage of vitamin A in rats fed carotene stereoisomers. *J. Nutr.* 103:20.
4. Stransky, H. and Schulze, I. (1977) Carotenoids in *Gallus domesticus*. Comparative analysis of blood and retina of chickens and egg yolk. *J. Comp. Physiol.* 115:265.
5. Neamtu, G., Salajan, G., Bilaus, C., Laszlo, T., Bodea, C., and Simpson, K. L. (1977) Research on the utilization of carotenoid pigments II. Effect of meal from the fruits of *Hyppophae rhamnoides* oil on the pigmentation of egg yolk. *Rev. roum. Biochem.* 14:109.
6. Puglisi, C. V. and deSilva, J. A. (1976) Determination of the carotenoid phytoene in blood by high-pressure liquid chromatography. *J. Chromatog.* 120:457.
7. Krinsky, N. I., Cornwell, D. G., and Oneley, J. L. (1958) The transport of vitamin A and carotenoids in human plasma. *Arch. Biochem. Biophys.* 73:233.
8. Bayfield, R. F., Falk, R. H., and Barrett, J. D. (1968) The separation and determination of  $\alpha$ -tocopherol and carotenoids in serum or plasma by paper chromatography. *J. Chromatog.* 36:54.
9. Bjornson, L. K., Kayden, H. J., Miller, E., and Moshell, A. N. (1976) The transport of  $\alpha$ -tocopherol and  $\beta$ -carotene in human blood. *J. Lipid Res.* 17:343.

Extraction of carotenoids from biological material usually consists of removing the carotenoid-containing lipids from the hydrophilic cell mass. Thus, the hydrocarbon carotenoids are often extracted with acetone, and the xanthophylls with the more polar

solvent, methyl alcohol. Nonpolar solvents, such as light petroleum or diethyl ether, are generally not used. Dried tissue often is better extracted if it is first rehydrated.

The pigments are usually transferred to nonpolar solvents (e.g., light petroleum, ethyl ether) by the addition of water. General references on extraction procedures have been published.<sup>1,2</sup>

The tissue for extraction should be fresh or stored at  $< -20^{\circ}\text{C}$ . Tissue may be lyophilized and stored under nitrogen but should not be air dried or dehydrated in solvents that would also extract carotenoids. Acetone, as well as other solvents,<sup>3,4</sup> have been used to extract carotenoids from feces and liver,<sup>1</sup> blood,<sup>1</sup> muscle,<sup>4</sup> and egg yolk.<sup>5</sup> In some cases antioxidants, e.g., pyrogallol, have been added to the extracting medium.

If acetone is used, it must be completely removed before the saponification step to prevent aldol condensation artifacts.<sup>3</sup> Samples should never be stored in acetone.

Extraction of liquids such as plasma or milk is greatly simplified since the extracting solvent coagulates the protein and leaves a clear upper layer. The extracting solvent can be separated from the precipitate by filtration, centrifugation or decanting off the upper layer. This procedure should be repeated two or three times. In some cases where the sample is high in lipid (milk, lard, etc.) the extraction is omitted and the sample is directly saponified.

The complete extraction of tissue usually requires homogenization with acetone, methanol, or similar solvent for an appropriate period of time in an electric blender. The resulting slurry is filtered through a sintered glass funnel or centrifuged. The process is repeated until complete extraction is obtained. Samples of less than 0.5 g may be extracted by being ground with washed sand and anhydrous sodium sulfate in a mortar. The resulting powder may be further extracted. The extracts are combined and washed into a non-polar solvent in a separatory funnel (Teflon stopcock). If necessary, the resulting repeatedly washed solution is concentrated on a rotary vacuum evaporator and dried over anhydrous sodium sulfate.

## Saponification

### References

1. Davies, B. H. (1976) Carotenoids. In: *Chemistry and Biochemistry of Plant Pigments*. Vol. 2, T. W. Goodwin, ed., London: Academic Press.
2. McLaren, D. S., Read, W. W. C., Awdeh, Z. L., and Tchalian, M. (1967) Microdetermination of vitamin A and carotenoids in blood and tissue. In: *Methods of Biochemical Analysis*. Vol. XV, Glick, ed., New York: Wiley.

The extraction procedure would be expected to remove carotenoids as well as unwanted lipid material. Saponification is necessary if individual pigments are to be isolated, in which case other lipids often interfere with the chromatographic separations. Some carotenoid esters have similar spectral and chromatographic properties as provitamin A compounds; these xanthophylls are readily separated from the carotenes on the basis of polarity. Saponification should be avoided when a carotenoid ester is to be analyzed, acetone is present, or in the few instances when the carotenoid is altered by saponification (e.g., astaxanthin  $\rightarrow$  astacene).

Milk and tissue<sup>2</sup> may be saponified directly with 5% KOH in absolute ethyl or methyl alcohol (1:4, milk:KOH solution; 1:8, tissue:KOH solution;  $75^{\circ}\text{C}$  for 25 minutes). Extracts

may be saponified by use of 5-10% KOH in methanol solution. The extracting solvent (light petroleum, ethyl ether, etc.) is removed either by heat or by a slow stream of nitrogen. The saponification is incomplete while phases exist."

The alkaline mixture may be heated on a steam table for 5-10 minutes in the dark under nitrogen, or kept in the dark at 5°C under nitrogen for 12-16 hours. Under nonoxidizing conditions, hot or cold saponification causes little loss of the carotenoids. Antioxidants may be added, but are usually not required. The pH should remain high during the saponification process.

The alkaline mixture is cooled and poured carefully into a Teflon separatory funnel containing an equal volume of light petroleum or ethyl ether. After extraction, water is added slowly and the lower, aqueous phase is discarded. The process is repeated until all of the KOH is removed. Sodium chloride or ethanol can be added to the separatory funnel to break emulsions that form.

## Chromatographic Separations

Total extractable carotenoids in foodstuffs and tissues can be estimated by determining the absorbancy of a lipid extract at 450-460 nm. This procedure, commonly used in association with nutritional methods for analyzing vitamin A, is useful quantitatively, i.e., whether extractable carotenoids are absent, present in small amounts, or abundant in the tissue or material examined. Clearly, no inferences can be made about the nature of the specific carotenoids present from a single absorbancy reading.

If information about specific carotenoids is desired, the carotenoid mixture must be separated into subgroups or pure compounds. Three ways of separating carotenoids are: (a) adsorption chromatography on columns of alumina, magnesium oxide, etc.; (b) thin-layer chromatography; and (c) HPLC analysis. Gas chromatography, which is very useful for separating many simple lipids, is not generally applicable to carotenoids.

### Alumina Column Chromatography

#### Reference

1. Krinsky, N. I., Cornwell, D. G., and Oncley, J. L. (1958) The transport of vitamin A and carotenoids in human plasma. *Arch. Biochem. Biophys.* 73:223.

#### Solvents

Light petroleum (30-60° bp)  
95% Ethyl alcohol  
Acetone, reagent grade

#### Supplies

Glass columns, test tubes, capillary tubes  
Spectrophotometer cells  
Alumina suitable for chromatography

## Instrument

Variable wavelength spectrophotometer

## Procedure: Serum/Plasma

1. Denature serum or plasma by the slow addition of an equal volume of 95% EtOH.
2. Extract carotenoids and vitamin A by the addition of two volumes of light petroleum followed by vigorous shaking.
3. Remove top layer by pipette and concentrate under vacuum or under a stream of nitrogen.
4. Prepare alumina by adding 6% water, stopper, shake and equilibrate for one hour.
5. Prepare chromatographic column by plugging with cotton or glass wool and pouring in water-deactivated alumina.  
Add pigment solution in a minimal amount of light petroleum. Develop the column with light petroleum to elute beta-carotene, alpha-carotene and less polar precursors and carotenoid esters. Lycopene and compounds with similar polarities (vitamin A esters) are eluted with light petroleum:acetone in a ratio of 96:4. Xanthophylls—lutein, zeaxanthin, vitamin A alcohol, etc.—are eluted with light petroleum:acetone in a ratio of 60:40.
6. Evaporate fractions to dryness under vacuum, add light petroleum and make up to a known volume.
7. Measure the absorbancy of a suitably diluted sample in a spectrophotometer.  
E<sub>1%<sup>1cm</sup></sub>—beta-carotene 2550 at 452 nm in hexane  
—lycopene 3450 at 472 nm in hexane  
—lutein 2550 at 445 nm in ethanol.
8. Calculate the amount of carotenoid present

In the example given below, the fraction eluted with light petroleum was made up to 25 ml. An aliquot of 0.6 ml was diluted to 3.0 ml with hexane and read at 453 nm in a 1 cm wide cuvette in a spectrophotometer. The absorbancy was 0.75. The amount of carotenoid in the fraction, expressed as mg all-*trans* beta-carotene, is given by the following formula:

$$\text{Amount} = \frac{A \times D \times \text{ml}}{\frac{E_{1\%}^{1\text{cm}}}{10}} = \frac{0.75 \times 5 \times 25}{\frac{2550}{10}} = 0.368 \text{ mg}$$

Where: A = absorbance  
D = dilution used to read on scale 0-1.0  
ml = total volume of sample

## Comments

The method gives a fair estimate of the total provitamin A content. Although the content is *expressed* as mg beta-carotene, other carotenoids of like polarity will also be present. Thus, the actual provitamin A content will depend on the proportion of beta-carotene in the mixture and the nature of the other polyenes present.

The provitamin A xanthophylls would be expected to elute in the more polar fractions. The advantages of this method are that it is quick, simple, and gives a much better estimate of provitamin A carotenoids than does the absorbance of the total extract.

# Microcolumn Technique Using Alumina

## Reference

1. McLaren, D. S., Read, W. W. C., Awdeh, Z. L. and Tchalian, M. (1967) Microdetermination of vitamin A and carotenoids in blood and tissue. In: *Methods of Biochemical Analysis*. Vol. XV, Glick, ed., New York: Wiley.

## Procedure

1. *Sample size*: 0.5 ml serum or plasma, 0.25 ml milk or 125 mg tissue.
2. *Extraction*: as above, concentrated to 5  $\mu$ l in light petroleum.
3. *Microcolumn*: capillary glass tube 1.5 mm  $\times$  150 mm plugged by a wire such as nichrome with asbestos or cotton. A test tube (250 mm) is filled with the tubes and alumina is added to the top of the test tube. The chromatographic columns are filled by vibration.
4. *Application of sample*: 5  $\mu$ l of light petroleum solution containing not more than 1 mg of carotene is applied to the asbestos or cotton plug end by a micropipette. A second 5  $\mu$ l of light petroleum is applied to move the pigments onto the adsorbent.
5. *Development*: the column is developed by placing it cotton plug side down in a test tube with 1 cm of solvent. The chromatogram is developed for 20 minutes in the dark.
6. *Identification*: individual bands may be removed by cutting the column with a file and crushing the section in a test tube. Appropriate solvent is added (acetone, chloroform, etc.) to the alumina, the contents mixed vigorously, the solvent extract removed with a Pasteur pipette, evaporated under vacuum, made up to a specific volume in an appropriate solvent and quantified in a spectrophotometer. The column does not separate alpha- and beta-carotene. In order to do so, the mixture must be re-chromatographed on MgO:Hyflo Supercel (1:2), using a solvent system of light petroleum:acetone (99:1).

The microcolumn method is suitable for small samples. The limitations and advantages of the Krinsky method described earlier in this chapter apply.

## Thin-layer Chromatography

### References

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## Procedure

The thin-layer chromatography technique has proved to be an invaluable method for the qualitative analysis, and identification of carotenoids and related compounds. It has served well in preliminary examination of extracts, small scale and preparative purifications, and identifications. An excellent book by Stahl<sup>1</sup> should be consulted for details.

TLC plates may be poured or obtained commercially from various companies. The plates are usually spread to a desired thickness with an active adsorbent and a binder (e.g., plaster of Paris, 0.5 mm). They are dried in an oven to activate them (e.g., Silica Gel 120°C for one hour) and then are kept dry before use. A line is drawn by pencil about 1.5 cm from the bottom edge. The plate is spotted (diameter <0.5 cm) with the saponified sample, dried and placed in a TLC chamber with an equilibrated solvent. The solvent is allowed to move about 10-15 cm up the plate. On the plate, the spots can be identified directly if they are colored, as in the case of the carotenoids, or can be made visible by a reaction with  $SbCl_5$  or iodine vapor.  $R_f$  values are calculated as the distance from the center of the spot to the starting line divided by the distance between the starting line and the solvent front. Table 8 gives a selected list of carotenoids and related terpenoids that have been separated by TLC.

TABLE 8.  
Selected  $R_f$  Values of Carotenoids and Related Compounds on TLC

Compound	System <sup>a</sup>			
	A	B	C	D
Beta-carotene	1.00	0.92	0.74	0.90-1.00
Retinyl esters				0.90-1.00
Retinal	0.65			0.69
Retinoic acid	0.08			0.08
Retinol				0.33
Beta-apo -8' -carotenal	0.67			
Beta-apo -10' -carotenal	0.71			
Alpha-carotene		0.96	0.80	
Lutein		0.65		
Zeaxanthin		0.39		
Cryptoxanthin		0.73		
Alpha-carotene			0.41	
Lycopene			0.13	

<sup>a</sup> System	Adsorbent	Eluant	Reference
A	Silica gel G	Acetone: petroleum ether (10:90)	3
B	$CaCO_3:MgO:Ca(OH)_2$ (30:6:4)	Petroleum ether:acetone:chloroform:methanol (50:50:40:1)	6,7
C	Magnesium oxide	Benzene:petroleum ether (90:10)	8
D	Silica gel G	Benzene:ethyl ether (90:10)	4

For quantitative work the spot should be scraped rapidly from the plate, the compound eluted with a suitable solvent, and the solution filtered through a small glass funnel fitted with nonabsorbent cotton. The choice of the solvent depends on the adsorbent and polarity of the compounds, e.g., diethyl ether, EtOH or acetone. Specific fractions are identified and quantified by UV/VIS spectra.

## Comment

Although carotenoids can be quickly destroyed by oxidation when left in a dry condition on a plate, Targan *et al.*<sup>4</sup> report a 97% recovery of beta-carotene standards and a high recovery of several retinoids under proper conditions. TLC has been effectively applied as well to the separation of retinyl esters from retinol (page 38). Because of the great differences in polarity among carotenoids, no single plate is suitable for all separations, as shown in Table 8. The advantages of the method are that it is simple, quick, and has the potential of being quantitative. It can also be used to assay the purity of fractions obtained from columns. The method has been successfully applied to extracts of plasma, serum and tissue.<sup>3,4,5</sup>

## High Pressure Liquid Chromatography

### References

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Of the methods mentioned above (i.e., column and TLC), none are as accurate or as suitable for multiple sample analysis as HPLC. Although the storage, extraction and saponification for all the methods can be identical, the advantage of HPLC is its resolution and speed of analysis. Several laboratories are reporting an analysis time of 15-20 minutes per sample from injection to quantification. Open-column chromatography, besides being time-consuming and allowing long-term exposure of the pigments to oxygen, light and adsorbents, often fails to resolve beta-carotene from other carotenes or its *cis* isomers. The *cis* isomer problem may be partly an artifact of the conventional separation and quantification procedure. The TLC method mentioned above lacks reproducibility, and accurate quantification can be prone to error due to the

instability of the carotenoid derivatives. The analysis of carotenoids by HPLC can be divided into two types: normal phase with a gradient elution, and reversed-phase with a non-gradient elution. In the latter method, the polar compounds come out first.

Stewart,<sup>1</sup> Fiksdahl, *et al.*<sup>2</sup> and Reeder and Park<sup>3</sup> separated carotenoid mixtures using MgO, silica and alumina, respectively, by gradient elution. Since gradient elution requires a potentially long equilibration period, is affected by the presence of water, and is influenced by changes in the mobile phase, it is not the most suitable procedure for routine sample analysis.

Successful separations of phytoene and beta-carotene from blood<sup>1</sup> and of lycopene, beta-carotene and alpha-carotene from tomato extracts<sup>5</sup> have been reported by use of a reversed-phase HPLC. Gerber *et al.*,<sup>6</sup> by modifying a published method,<sup>5</sup> have analyzed lycopene, alpha- and beta-carotene and retinyl esters in human serum. A review of HPLC procedures for carotenoids, as well as other physical methods, has recently appeared.<sup>7</sup>

## Apparatus

Centrituge

Column C-18 (Zorbax ODS, Whatman Partisil - PXS-5/ODS or equivalent)

HPLC system equipped with variable wavelength detector preferably with stop-scan capability

Recorder

Hamilton 710 syringe or equivalent

Guard column

Vortex mixer

## Reagents

Acetonitrile, nanograde

Methyl alcohol, absolute

Chloroform, reagent grade

Carotenoid standards: beta-carotene, alpha-carotene, lycopene, lutein, beta-cryptoxanthin (Sigma or equivalent)

Retinol

Retinyl palmitate

## Extraction and Analysis

*Blood.*<sup>6</sup> Pipette 100  $\mu$ l serum into a polyethylene microcentrifuge tube. Add 100  $\mu$ l absolute methanol and vortex for 15 seconds. Extract mixture with 200  $\mu$ l CHCl<sub>3</sub> by vortexing for 60 seconds and centrifuging for 10 minutes. Inject 50  $\mu$ l of bottom layer into HPLC.

*Tissue.*<sup>5</sup> Extract and saponify as above. Place purified extract in the freezer at  $<-10^{\circ}\text{C}$  overnight to precipitate steroids if present. Inject sample through a C-18 guard column to protect the main column.

*Quantification.* Refer to sections beginning on pages 14 and 40.

### Chromatographic conditions.

Solvent	ACN:CHCl <sub>3</sub> *	ACN:CHCl <sub>3</sub> *	MeOH:ACN:CHCl <sub>3</sub> †
Solvent ratio	92:8	92:8	46:46:8
Elution times (min):			
Retinyl palmitate			13.3
Beta-carotene	13.2	20.0	13.3
Alpha-carotene	12.4	18.3	12.4
Lycopene	7.8	10.4	8.5
Column	Partisil-5/ODS	Zorbax/ODS	Zorbax/ODS
Flow rate	2.0 ml/min	2.0 ml/min	2.0 ml/min
Detection wavelength	470 nm	466 nm	325/466 nm
Reference	5	6	6

\*Acetonitrile:chloroform

†Methanol:acetonitrile:chloroform

### Comments

Similar results were obtained with MeOH substituted for part of the acetonitrile, at some saving in costs. For the determination of retinyl esters and beta-carotene, two chromatographic runs are required because of their similar retention times. For one run, retinyl esters may be detected at 325 nm and for the other carotene at 466 nm. The detection limits are 0.00395, 0.0372 and 0.0285 nmoles for lycopene, alpha-carotene and beta-carotene, respectively.<sup>5</sup> Recovery of added retinyl palmitate, beta-carotene, alpha-carotene and lycopene was 88, 98, 94 and 94%, respectively.<sup>6</sup>

The HPLC method of analysis of carotenoids is still in a developmental stage inasmuch as the separation of only a few carotenoids has been reported. Much work needs to be done with other provitamin A and non-provitamin A carotenoids. The relative retention times of *cis-trans* isomers also need to be studied. Finally, HPLC analysis, while perhaps the best method, requires skilled technicians and expensive equipment and solvents.

# Recommendations and Research Needs

The present document deals primarily with methods which are useful in measuring vitamin A in serum and tissues. Clearly, each of these methods might be improved, or indeed new procedures which are simpler, more rapid and more specific might be developed. Particularly in regard to the carotenoids, such procedures should be developed. Despite the utility of all of the methods cited, each gives a value dependent upon the specific characteristic of vitamin A being measured and is confounded by different contaminants in the biological extract. Thus, careful comparisons of methods when applied to different tissues or under different circumstances of sample collection and storage is well warranted. Even in the use of a single method, annoying discrepancies exist, e.g., whether measured serum vitamin A values in frozen samples increase with the time of storage or not when the colorimetric assay is used. All vitamin A values collected in a survey are ultimately expressed in terms of some presumed standard which, in all cases but the UV inactivation method, requires a vitamin A preparation. Although retinyl acetate in cottonseed oil is very useful, the availability of a highly stable reference standard in a form convenient for use in nutritional laboratories throughout the world would be most welcome.

Quite apart from the methodology itself is its application to key issues of vitamin A nutriture. Certainly of major interest is the development of methodology for more effectively assessing marginal and adequate states of vitamin A nutriture in individuals and populations. Along similar lines, a deeper understanding of nutritional and physiological factors influencing the storage of vitamin A in the liver and other tissues, and its transport to and uptake by target tissues would be most gratifying. Inasmuch as the newborn child possesses very limited vitamin A reserves, the mechanism of transfer of vitamin A and carotenoids from the maternal circulation into the breast milk, and across the placenta and into the fetus should be explored. The importance of the mother's nutritional status in regard not only to vitamin A but also to other nutrients should be probed. Although the concentrations of both vitamin A and fat in milk are known to increase as the breast empties during suckling, many aspects of the process are not well defined, e.g., effects of suckling frequency, diurnal variation, relation to maternal intake of a vitamin A-rich meal. And finally, the relationship of vitamin A concentration in the milk as a function of the length of the breast feeding period, weaning practice and the age of both infant and mother has not been well defined. Clearly, information drawn from such studies would allow us to interpret the data obtained by the methods described in this manual in a more knowledgeable way.

In summary, the recommendations are:

- a highly stable reference standard for vitamin A, in a form convenient for nutrition

laboratories, should be made available worldwide;

- proper sample handling and quality control under actual survey conditions should be monitored in all vitamin A surveys;
- discrepancies relative to the sample handling or analysis of vitamin A by a given method which appear in the literature should be explored and resolved;
- the results obtained by commonly used survey methods for vitamin A analysis should be compared under standardized conditions and at periodic intervals;
- new procedures should be developed for the analysis of specific carotenoids in serum, tissues and foods;
- new simplified procedures for use under field conditions should be developed for determining provitamin A in serum, tissues and food;
- procedures should be developed for determination of the biological availability of provitamin A substances in food;
- new methods for the quantitative evaluation of marginal and adequate states of vitamin A nutriture should be explored;
- the physiological impact of a marginal state of vitamin A nutriture should be probed;
- the possible physiological and nutritional significance of the ratio of apo- to holo-RBP in the plasma should be studied;
- appropriate guidelines of vitamin A nutriture, which are specific for children of different ages, should be developed; and
- further research studies on the physiological basis for nutritional assessment should be undertaken. These should include:
  - factors, both nutritional and physiological, which affect the liver storage, plasma transport, tissue uptake and recycling of vitamin A;
  - the mechanisms of vitamin A transfer from the maternal blood to the milk, and maternal nutritional factors affecting the process;
  - the concentration of vitamin A and specific carotenoids in the milk as a function of the milk remaining in the breast, suckling frequency, etc.; and
  - the concentration of vitamin A and specific carotenoids in the milk as a function of length of lactation, weaning practice, mother's nutritional status, and the development of vitamin A deficiency in the infant.